



PHD

Lipid Phosphatases and Oxidative Signalling in T Lymphocytes

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Lipid Phosphatases and Oxidative Signalling in T Lymphocyte Biology

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A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Pharmacy and Pharmacology

June 2015

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Abstract

Adaptive immune responses are highly co-ordinated and rely upon efficient intracellular communication to orchestrate cell function. Phosphoinositide 3-kinase (PI3K) signalling is a well-studied and important positive mediator in T lymphocyte function; however the role for SH2-domain containing inositol phosphatase 1 (SHIP-1), a negative regulator of PI3K signalling, has not been so thoroughly investigated. The use of knockout mouse models has given an insight into the role of SHIP-1 in murine T cells, but these are compromised by loss of function during development which impinges upon mature T cell function and by the loss of non-catalytic functions of SHIP-1.

Recent work has indicated a clear role for reactive oxygen species (ROS), specifically hydrogen peroxide (H_2O_2), in immune cell signalling and functional responses including migration. However like SHIP-1, the functional roles of ROS are poorly understood in human T lymphocytes, particularly the mechanisms by which ROS signals to alter T lymphocyte biology. ROS has been previously shown to activate PI3K, Mitogen activate protein kinase (MAPK) and Src family tyrosine kinase (SFK) signalling in a number of different cell types. In addition, ROS have been shown to inactivate phosphatase and tensin homology (PTEN), another negative regulator of PI3K, and are postulated to inactivate SHIP-1 signalling.

A pharmacological approach was utilised to manipulate the catalytic activity of SHIP-1 and the cellular accumulation of ROS in primary human T lymphocytes. Remarkably, it was determined that both SHIP-1 activation and inhibition reduced the ligand-mediated functions of human T lymphocytes, including signalling, proliferation, adhesion and migration. Furthermore, H_2O_2 selectively inhibited directional migration to chemokine CXCL11, enhanced F-actin polymerisation and decreased actin polarisation to CXCL11. H_2O_2 required SFK signalling to induce the phosphorylation/catalytic activation of SHIP-1 and to decrease the surface expression of CXCR3, both of which could be mechanisms underlying the deficiency in migration observed with H_2O_2 .

Abbreviations

Abbreviations	Definition
3AC	3 α -aminocholestane
Akt/PKB	Protein kinase B
APCs	Antigen presenting cells
BCR	B cell receptor
CD	Cluster of differentiation
CD4+	Helper T lymphocytes
CD8+	Cytotoxic T lymphocytes
CdtB	Cytolethal distending toxin subunit B
CFSE	Carboxyfluorescein succinimidyl ester
Chk	Csk homology kinase
CLL	Chronic lymphocytic leukemia
CRAC	Ca ²⁺ release activated Ca ²⁺ channel
Csk	C-terminal Src kinase
DAG	Diacylglycerol
DAMPS	Damage associated molecular patterns
DARC	Duffy antigen receptor for chemokines
DCFDA	2',7'-dichlorofluorescein diacetate
DN	Double negative
DP	Double positive
DUOX	Dual oxidase enzymes
ELR	Glutamic acid leucine arginine
ER	Endoplasmic reticulum
FAK	Focal adhesion kinase
GBM	Glioblastoma multiforme
GPCR	G-protein coupled receptor
GRK	G protein coupled receptor kinase
H ₂ O ₂	Hydrogen peroxide
HEV	High endothelial venules
ICOS	Inducible co-stimulatory receptor
IFN	Interferon
IL	Interleukin
iNHL	Indolent non-Hodgkin lymphoma
IP3	Inositol triphosphate
IPF	Idiopathic pulmonary fibrosis
ITAMs	Immunoreceptor tyrosine-based activation motifs
ITIM	Immunoreceptor tyrosine-based inhibition motifs
LFA-1	Lymphocyte function associated antigen 1
MAPK	Mitogen activate protein kinase
MCL	Mantle cell lymphoma
MM	Multiple myeloma
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NHL	Non-Hodgkin lymphoma
NK	Natural killer
NOX	NADPH oxidase family

NSAID	Non-steroidal anti-inflammatory drugs
PAMPS	Pathogen associated molecular patterns
PH	Pleckstrin Homology
PI(3,4)P2	Phosphoinositol (3,4) bis-phosphate
PI(3,4,5)P3	Phosphoinositol (3,4,5) tris-phosphate
PI(4,5)P2	Phosphoinositol (4,5) bis-phosphate
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PTEN	Phosphatase and tensin homology
PTKs	Protein tyrosine kinases
RCC	Renal cell carcinoma
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute (cell growth medium)
RSH	Reducing thiols
S1P	Sphingosine-1-phosphate
SDF1	Stromal development factor 1
SEB	Staphylococcal enterotoxin B
SEM	Standard error of the mean
SFK	Src family tyrosine kinase
SH	Src homology domain
SHIP-1	SH2-domain containing inositol phosphatase 1
SHP1	SH2-domain containing tyrosine phosphatase 1
SLL	Small lymphocytic lymphoma
SLO	Secondary lymphoid organs
SOD	Superoxide dismutase
Syk	Spleen tyrosine kinases
TCR	T cell receptor
TLR	Toll-like receptor
VCAM-4	Vascular cell adhesion molecule-4
VLA-4	Very late antigen-4
ZAP-70	Zeta-chain associated kinase 70kDa

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CHAPTER 1: Introduction

1.1 Overview of the immune system

We depend upon our immune system's ability to produce a co-ordinated and rapid response to protect us from foreign pathogens and tissue damage resultant of infection or trauma. To function correctly, the immune system has evolved to be complex, multi-cellular and destructive in nature. As such, it is critical that our immune system is highly regulated and targeted. Dysregulation of the immune system results in a wide array of pathologies; under-activity increases our susceptibility to infection, whilst over-activity can lead to chronic inflammation and autoimmune disease.

The immune system is commonly divided into two complementary branches; the immediate response is termed the innate immune system and the later more specific response is termed the adaptive immune system. The cells of the immune system are shown in Figure 1.1.

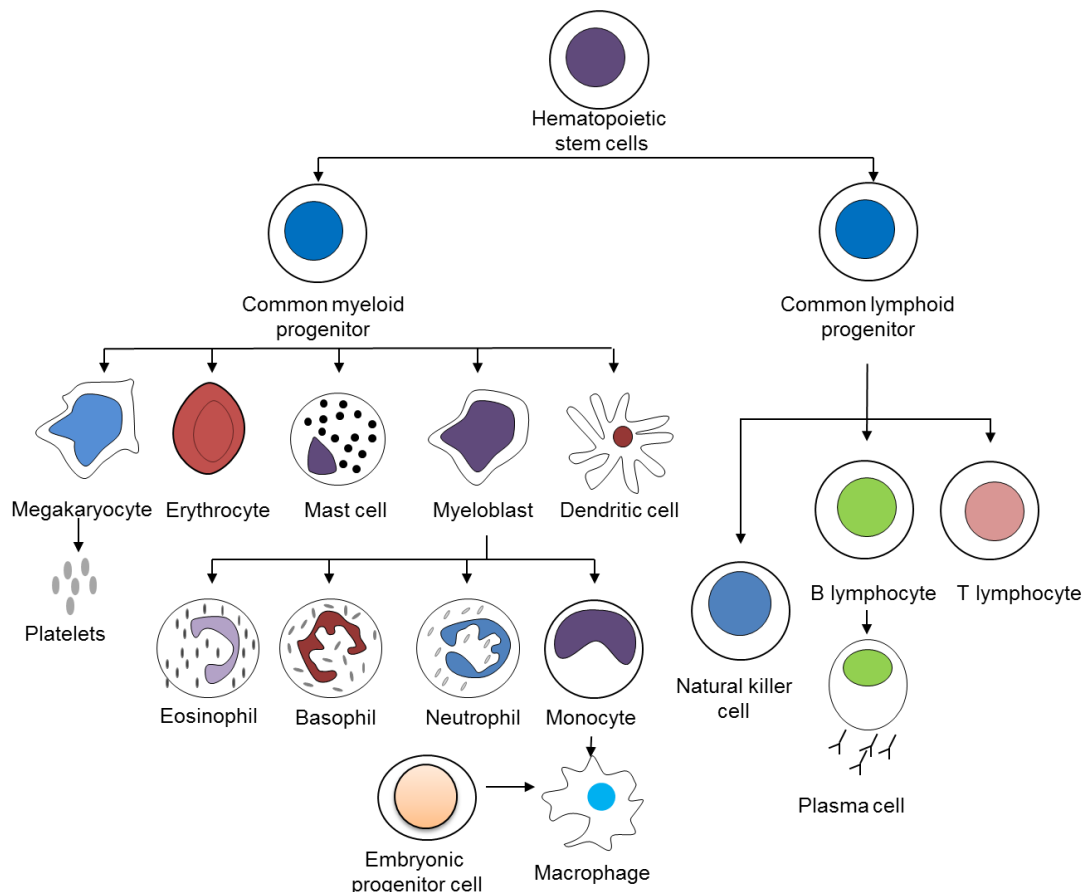


Figure 1.1 Cells of the immune system. The majority of the cells of the immune systems differentiate from a common hematopoietic stem cell progenitor. Two main branches are generated; the left branch is the myeloid cells and right branch represents the lymphoid cells. Although monocytes have long been described as the sole precursors of macrophages, recent evidence suggests that a significant number of tissue resident macrophages arise prenatally during embryonic development from progenitors derived from the yolk sac or fetal liver (Hettinger *et al.*, 2013) (Original graphic).

1.1.1 The innate immune system

The innate immune system is the first method of defence against a pathogen. Sequentially it limits pathogen entry, rapidly recruits immune cells to site of infection, inactivates or destroys invaders and then repairs the damaged tissue. The skin and epithelial cell linings of the lungs and gut are important anatomic barriers which normally act to prevent pathogen entry to the body. However, when these tissues become infected or injured an acute inflammatory response is initiated to protect the host. Briefly, tissue resident inflammatory cells become activated when they detect damage associated molecular patterns (DAMPS) or pathogen associated molecular patterns (PAMPS) produced by the damaged cells or pathogens, respectively. Tissue resident immune cells include mast cells which de-granulate and release inflammatory mediators including histamine, prostaglandins and leukotrienes which enhance inflammation, vasodilation and vascular permeability. This allows increased leukocyte recruitment from the blood to amplify the immune response. Phagocytic cells (macrophages and neutrophils) engulf debris and foreign bodies to neutralise or destroy the pathogen. Finally, tissue resident dendritic cells capture antigen and enter the lymph to co-ordinate activation of the adaptive immune system (Delves *et al.*, 2000b; Medzhitov *et al.*, 2008) (Figure 1.2).

1.1.2 The adaptive immune system

The adaptive immune system provides a highly specific response to a specific pathogen. Adaptive immunity can also provide long-lasting protection: and an immunological memory to a specific pathogen, co-ordinating a more effective immune response upon subsequent exposure. To do this each adaptive immune cell specifically recognises only one antigen, which results in its initial response taking days to develop. Antigens are transported to the lymphoid organs by antigen presenting cells (APCs) where they are recognised by the adaptive immune cells, the naïve B-lymphocytes and T-lymphocytes. A humoral response is driven by B lymphocytes. Activation of B lymphocytes induces proliferation, maturation and the secretion of antibodies. Antibodies bind/block the toxicity of the pathogen and target it for phagocytosis. Whereas T lymphocytes drive cell mediated immunity, so called due to the T lymphocytes ability to detect intracellular pathogens such as viruses by recognising antigens presented upon the surfaces of other cells (Figure 1.2). Activated T lymphocytes are divided into cytotoxic (CD8+) and helper (CD4+) subsets. Both subsets subsequently proliferate, with CD8+ able to destroy the pathogen through the release of cytotoxins and CD4+ able to differentiate into effector cells which orchestrate

an inflammatory response against the pathogen (Delves *et al.*, 2000a; Pepper *et al.*, 2011). The T lymphocytes will be discussed further in following sections.

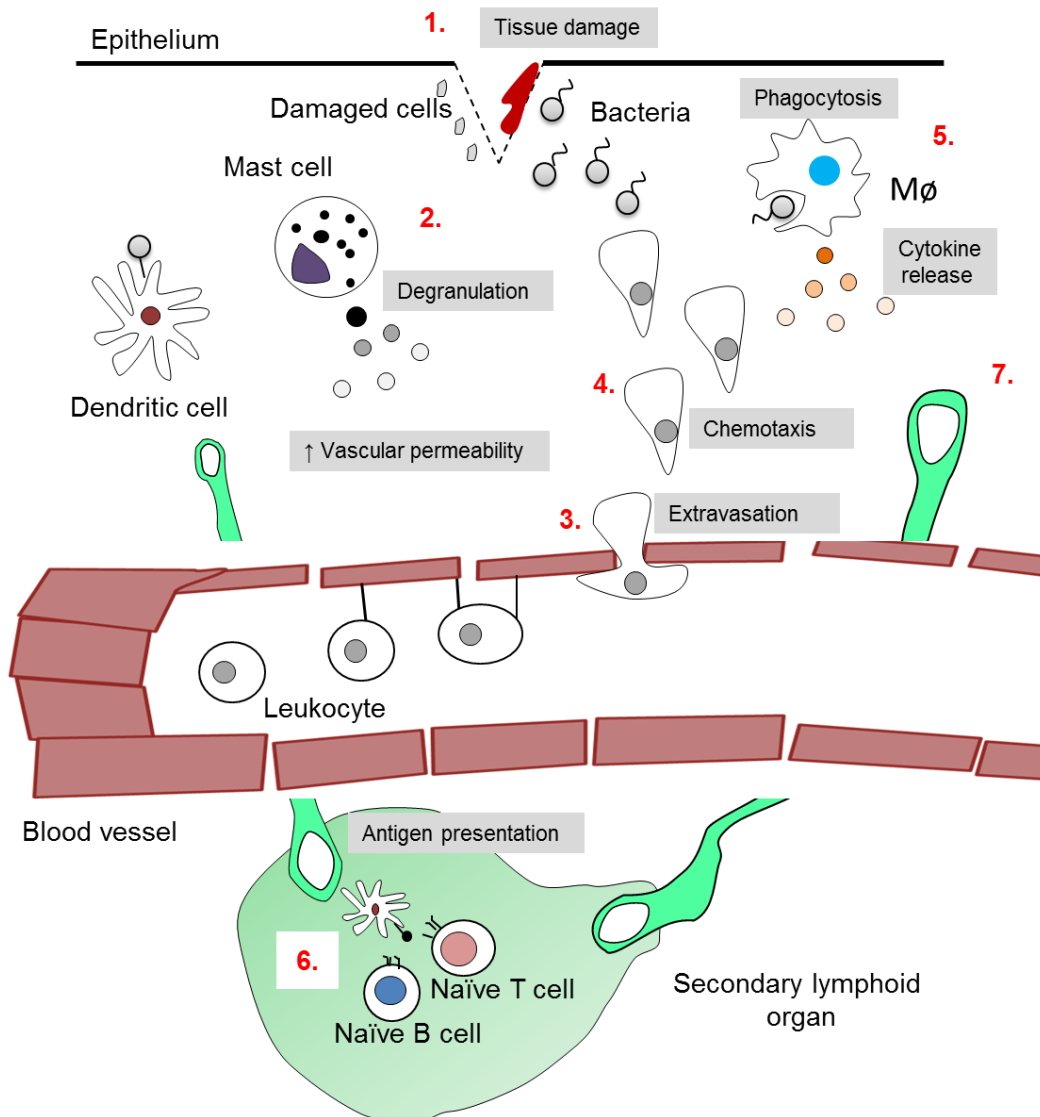


Figure 1.2 The immune response. 1) The epithelium provides an anatomical barrier preventing pathogen entry; but following epithelial wounding, damage or infection tissue resident inflammatory cells are activated. 2) Upon activation, macrophages and mast cells release of inflammatory mediators, such as chemokines and cytokines to evoke vasodilation, increase vascular permeability and promote expression of adhesion receptors on the endothelium of blood vessels. 3) Leukocytes are recruited from the blood vessels, involving tethering, rolling, adhesion, and extravasation into the tissue. 4) Leukocytes migrate towards the source of inflammation following gradients of chemoattractant. 5) Phagocytic cells (macrophages and neutrophils) engulf debris and foreign bodies to neutralise or destroy the pathogen. 6) Tissue resident dendritic cells capture antigen and enter the lymph to co-ordinate activation of the naïve T and B lymphocytes of the adaptive immune system. 7) Activation of lymphocytes causes their differentiation, clonal expansion and migration to the site of infection. Lymphocytes are part of the adaptive arm of the immune response, and induce cell-mediated (cytotoxic) and humoral (B cell antibody mediated) killing of pathogen. Finally, infection is resolved and wound is repaired (Original graphic).

1.1.3 Pathologies of immune cell regulation

Pathologies of inflammatory regulation include 1) an overly abundant acute inflammatory response to pathogen (*i.e.* cytokine storm), 2) primary deficiencies in immune cells (*i.e.* Kostmann syndrome or chronic granulomatous disease) or 3) the inability of the inflammatory response to eradicate the primary initiator of the immune response, resulting in sustained inflammation and tissue damage (Tabas *et al.*, 2013).

Autoimmunity belongs to the third category, whereby most commonly the primary stimulator of the immune response is the body's own cells which the immune system has lost the ability to recognise. As such, the immune system continually attempts to destroy the body's own tissues. Autoimmune diseases arise through aberrant activation of the T and B lymphocytes driving a continuous inflammation state (Arkwright *et al.*, 2002).

Autoimmune diseases are often associated with life changing, painful symptoms. There are more than eighty illnesses known to be caused by autoimmunity which most commonly affect women and are among the top ten leading causes of death in women under the age of 65. Current treatment aims to reduce the inflammation by using broad spectrum immunosuppression (*i.e.* Non-steroidal anti-inflammatory drugs (NSAID) and steroids), which can sometimes dangerously relieve sufferers of a physiologically functioning immune system. As such there is a huge drive for a more targeted treatment which functions to suppress the ability of lymphocytes to drive inflammation in the first place (Walsh *et al.*, 2000).

Ideally, an autoimmune and inflammatory disease treatment would alter the threshold of immune activation to restore homeostasis, modulate antigen-specific responses to spare target organs and reduce immune cell migration to inhibit the enhanced recruitment. Therefore in order to create new treatments to target the immune system, a detailed understanding of the signalling pathways involved in lymphocyte functions is required.

1.2 T lymphocytes

1.2.1 T lymphocyte development and maturation

T lymphocytes originate from hematopoietic stem cells (HSCs) in the bone marrow which home to the thymus to mature (Miller, 2002). Development in the thymus requires a stringent selection process, where thymocytes mature from double negative ($CD4^-/CD8^-$) to either a singular positive $CD4^+$ or $CD8^+$ cell which expresses a self-restricted $\alpha\beta$ TCR receptor (Figure 1.3) (Anderson *et al.*, 2001). $CD4^+$ and $CD8^+$ T lymphocytes bind to antigen upon either Class II or Class I MHC molecules respectively (Germain, 1994). During development, gene rearrangement of the variable (V), diversity (D), joining (J) and constant (C) regions of the TCR receptor produces a plethora of structurally distinct TCR receptors, which allow T lymphocytes to recognise specific antigens.

Singular positive $CD4^+$ and $CD8^+$ cells are long lived and can remain in the extrathymic environment for weeks or months. These naïve (prior to stimulation with antigen) T lymphocytes typically express low levels of glycoprotein CD44 and high levels of lymph node homing receptors CD62L and CCR7. Naïve lymphocytes survey the lymphatic system for antigen and rely on contact with self-peptides and IL-7 to survive and proliferate. Following activation, IL-7 can also drive the transition of naïve and effector T lymphocytes to form antigen specific memory T lymphocytes, cells either become central memory T lymphocytes ($CD44^{hi}$, $CD62L^{hi}$, $CCR7^{hi}$) or effector memory cells ($CD44^{hi}$, $CD62L^{low}$, $CCR7^{low}$) (von Andrian *et al.*, 2003).

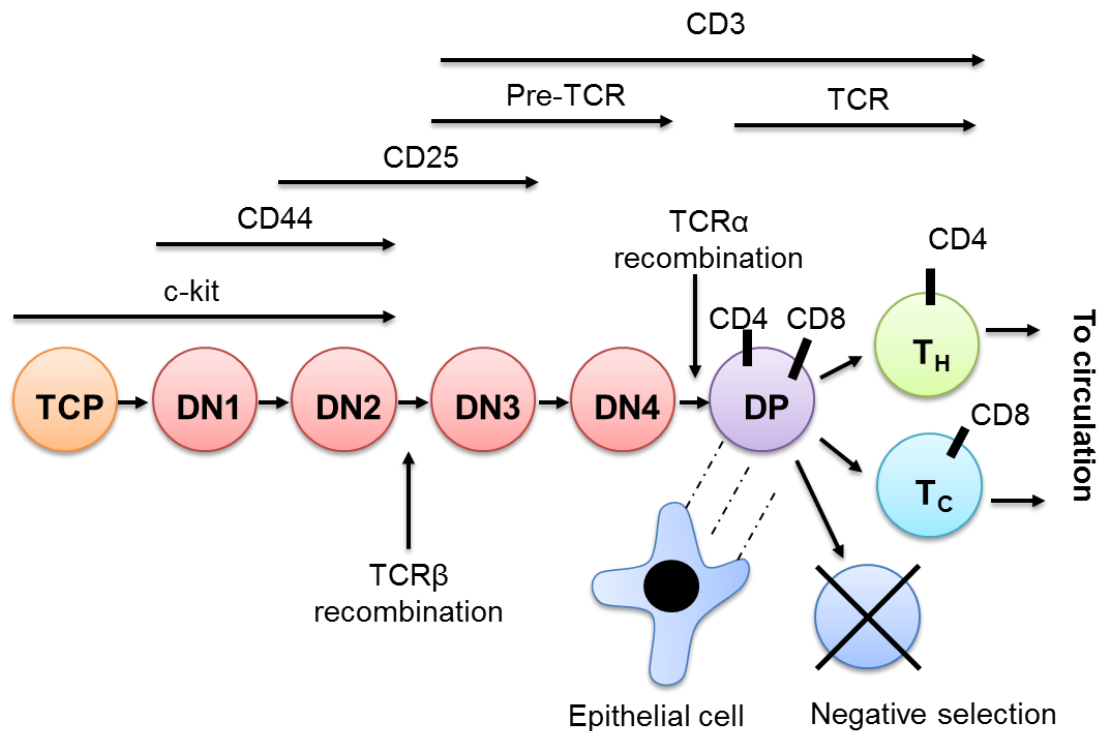


Figure 1.3 T lymphocyte maturation in the thymus. T lymphocyte precursors (TCPs) migrate to the thymus from the bone marrow. These cells are termed double negative (DN) as they do not express either of the T cell cluster of differentiation (CD) markers CD4 or CD8. T lymphocyte maturation involves distinct phases that are marked rearrangement of the TCR receptor, as well as changes in the expression of cellular surface proteins. $c\text{-kit}^+$ and CD44^+ DN1 thymocytes progress to the DN2 stage by gaining CD25^+ expression and down-regulating CD44 expression. DN2 thymocytes also begin to rearrange the $\text{TCR}\beta$ subunit of the TCR receptor and the cells lose their pluripotent ability to transform into B lymphocytes or natural killer cells. At the DN2 stage, the TCR components begin to be rearranged forming either DN3 or T lymphocytes which eventually express a $\gamma\delta$ TCR receptor. $c\text{-kit}^-$, CD44^- , CD25^+ DN3 thymocytes, express $\text{TCR}\beta$ and a pre- $\text{TCR}\alpha$ in a complex with CD3. CD25 expression is lost at the DN4 stage and cells rearrange the $\text{TCR}\alpha$ receptor and express CD4^+ and CD8^+ to become double positive (DP). The DP cells that bind to MHC expressed on epithelial cells gain signals to progress to singular positive CD8^+ or CD4^+ cells, this is termed positive selection. A stringent selection process occurs whereby T lymphocytes are screened for TCR reactivity to self-peptides bound to MHC molecules. Cells with high avidity for the peptides undergo apoptosis as they will be self-reactive, this is termed negative selection. However, about 98% of DP cells have very low affinity for the MHC presented peptide and die from “neglect” or lack of a TCR signal, termed positive selection. The surviving singular positive CD4^+ or CD8^+ cells leave the thymus and enter the circulation (Godfrey *et al.*, 1993) (Original graphic).

1.2.2 T cell activation

The activation of T lymphocytes is described by a two signal model. Firstly, “signal 1” involves the TCR engaging with a specific foreign peptide (antigen) presented upon the major histocompatibility complex (MHC) of an APC. The MHC protein is also bound by either CD4 or CD8 on the cell. Secondly, “signal 2” is initiated by the binding of costimulatory molecules on the surface of the T cell, typically activation of CD28 by an appropriate ligand such as CD80/CD86 (B7.1 or B7.2). B7.1 and B7.2 are up-regulated upon the surface of APCs following inflammatory cytokine exposure. The lack of co-stimulation through CD28 commonly results in T lymphocyte anergy (McAdam *et al.*, 1998; Sharpe *et al.*, 2006), a protective mechanism which prevents aberrant response to self-antigen (Figure 1.4).

In addition to CD28 ligation, other molecules can also provide the co-stimulatory signal to activate T lymphocytes. These include the inducible co-stimulatory receptor (ICOS), which is expressed on activated T lymphocytes, lymphocyte function associated antigen 1 (LFA-1) and some $\beta 1$ integrins including very late antigen-4 (VLA-4) (Van Severter *et al.*, 1990) [Figure 1.4]. The ICOS receptor shares a high level of homology with CD28, but exhibits a distinct expression pattern and signalling profile which indicates non-redundant functions. ICOS activation is often associated with the activation of follicular T helper cells and Treg cells (Ma *et al.*, 2012).

Negative regulation of TCR signalling also occurs. B7.1 or B7.2 can also inhibit T cell activation by binding to inhibitory co-receptors such as cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death 1 (PD1) (Figure 1.4). Other B7 family members, B7.3 and B7.4 have also been observed, however at present their binding partners and roles in T lymphocyte activation are not well understood (Okamoto *et al.*, 2003). CTLA-4 is upregulated upon activated T lymphocytes and is important in the resolution of the adaptive immune response (Oosterwegel *et al.*, 1999; Smith-Garvin *et al.*, 2009). Termination of TCR signalling also occurs through negative regulators such as phosphatase and tensin homolog (PTEN) or the Dok adaptor proteins of SH2-domain containing tyrosine phosphatase 1 (SHP1) (Smith-Garvin *et al.*, 2009). Super-antigens, such as staphylococcal enterotoxin B (SEB) act independently of MHC to activate a large number of T lymphocytes irrespective of the antigen specificity of these cells (Alber *et al.*, 1990).

The TCR receptor complex does not have intrinsic enzymatic activity and relies on recruitment of cytoplasmic signalling molecules for propagation of the receptors signal. The CD3 complex contains immunoreceptor tyrosine-based activation motifs (ITAMs)

which provide docking sites for the recruitment of signalling molecules containing SH2 domains. Signal transduction downstream of the TCR receptor involves multiple kinases and Ca^{2+} elevation followed by changes in gene transcription which are discussed further Section 1.4. The signalling has a number of crucial roles in T lymphocyte activation, perhaps the most important of which is the production of IL-2. IL-2 is a key factor in the activation and proliferation of T lymphocytes (Smith-Garvin *et al.*, 2009).

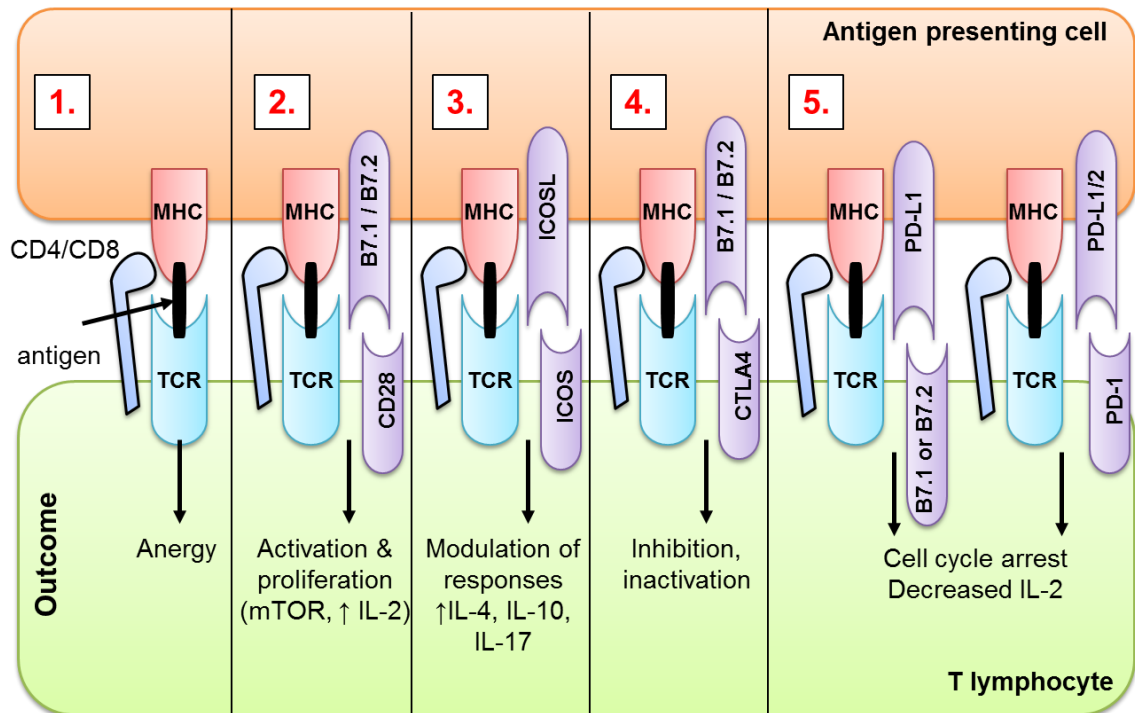


Figure 1.4 T cell activation and co-stimulation. “Signal 1” consists of the recognition of peptide antigen presented upon MHC complexes by the TCR receptor. CD4 T lymphocytes recognise Class II MHC which are expressed upon professional APCs, whereas CD8 T lymphocytes recognise Class I MHC which is expressed upon all cells. **1)** “Signal 1” alone induces anergy, or even death of the T lymphocyte. **2)** However, a combination of “signal 1” and “signal 2”, provided by B7.1 or B7.2 on the APC binding CD28, directs the cell towards activation and proliferation (e.g. IL-2 production). **3)** Upon T cell activation, inducible co-stimulatory receptor (ICOS) is upregulated and interacts with ICOSL to modulate the functions of the active cells. ICOS is highly homologous with CD28, yet exhibits key differences in signalling and expression. ICOS has a minimal role during naïve T lymphocyte priming or initial proliferation. However, in activated cells ICOS has been shown to induce greater PI3K signalling and is crucial in production of IL-21 and IL-4, key cytokine in the development of T follicular helper cells (Okamoto *et al.*, 2003). ICOS also has a role in the function and homeostasis of Tregs (Tuettenberg *et al.*, 2009). **4)** Negative regulation of TCR signalling occurs through the interaction of B7.1 or B7.2 within CTLA4 or **5)** via Programmed Death Ligand (PD-L) co-stimulation.

1.2.3 T lymphocyte differentiation and subsets

The differentiation of naïve T lymphocytes into effector and memory cells is fundamental in T lymphocyte mediated cellular immunity. Activated cytotoxic CD8+ cells are very effective at direct lysis of infected or malignant cells which express antigen upon their cell surface, whereas helper CD4+ cells produce cytokines to stimulate other immune cells and mobilise a powerful inflammatory response. Most effector cells will disappear after the antigenic agent is destroyed, although some remain and form memory cells, which are able to perform immediate effector functions if re-exposed to the same antigen.

CD4⁺ T helper cells acquire highly specialised functions by differentiating into distinct subsets. Differentiation is co-ordinated by the external cytokine environment and activation of specific transcription factors. CD4+ T helper cell lineages are categorised by their distinct surface receptor expression and cytokine production (Figure 1.5). Briefly, Th1 cells produce cytokines involved in activating innate immune cells and B lymphocyte class switching to greatly enhance their ability to kill intracellular pathogens. Th1 cells are implicated in the pathology of multiple sclerosis (Jager *et al.*, 2009). Whereas, Th2 cells are required for immunity against an extracellular pathogen (i.e. helminths), and are able to regulate B lymphocyte activation and antibody class-switching to aid eosinophil recruitment and promote mucosal defence. Aberrant Th2 responses are implicated in allergic diseases such as asthma (Zhu *et al.*, 2010).

Th17 cells arrive early at sites of inflammation and regulate neutrophil recruitment and activation. They are commonly found at epithelial barrier sites and therefore are thought to be involved in fighting extracellular bacteria and fungi (Korn *et al.*, 2009). Th17 cells produce IL-17A, IL-17F, IL-21 and IL-22. IL-17A and IL-17F have both been implicated in airway inflammation (Liang *et al.*, 2007; Fouser *et al.*, 2008), as well as several autoimmune diseases including multiple sclerosis, psoriasis, inflammatory bowels disease, rheumatoid arthritis and systemic lupus erythromatosis (Fouser *et al.*, 2008; Shin *et al.*, 2011; Pan *et al.*, 2013). In addition, they are also thought to have both pro- and anti- tumour functions, with IL-17F exacerbating tumour growth by facilitating angiogenesis (Murugaiyan *et al.*, 2009; Coffelt *et al.*, 2015; Saito *et al.*, 2015).

Tregs can be broadly divided into natural Treg (nTregs), which are generated in the thymus, and inducible Tregs (iTregs), which can be generated from naïve T lymphocytes in the periphery (Zheng *et al.*, 2004; DiPaolo *et al.*, 2007). Tregs aid immunological self-tolerance by suppressing self-reactive T lymphocytes (Wing *et al.*,

2010). Further T lymphocyte subsets Th9 and Th22 have more recently been identified, which produce IL-9 and IL-22 respectively. The niche functions of these cells are currently being investigated, IL-9 is known to exhibit pleiotropic functions and has putative roles in pathogen immunity and immune-mediated disease (Kaplan *et al.*, 2015). IL-22 regulates auto-antibody production and restricts commensal bacteria to their tissue niches preventing inflammation (Raphael *et al.*, 2014).

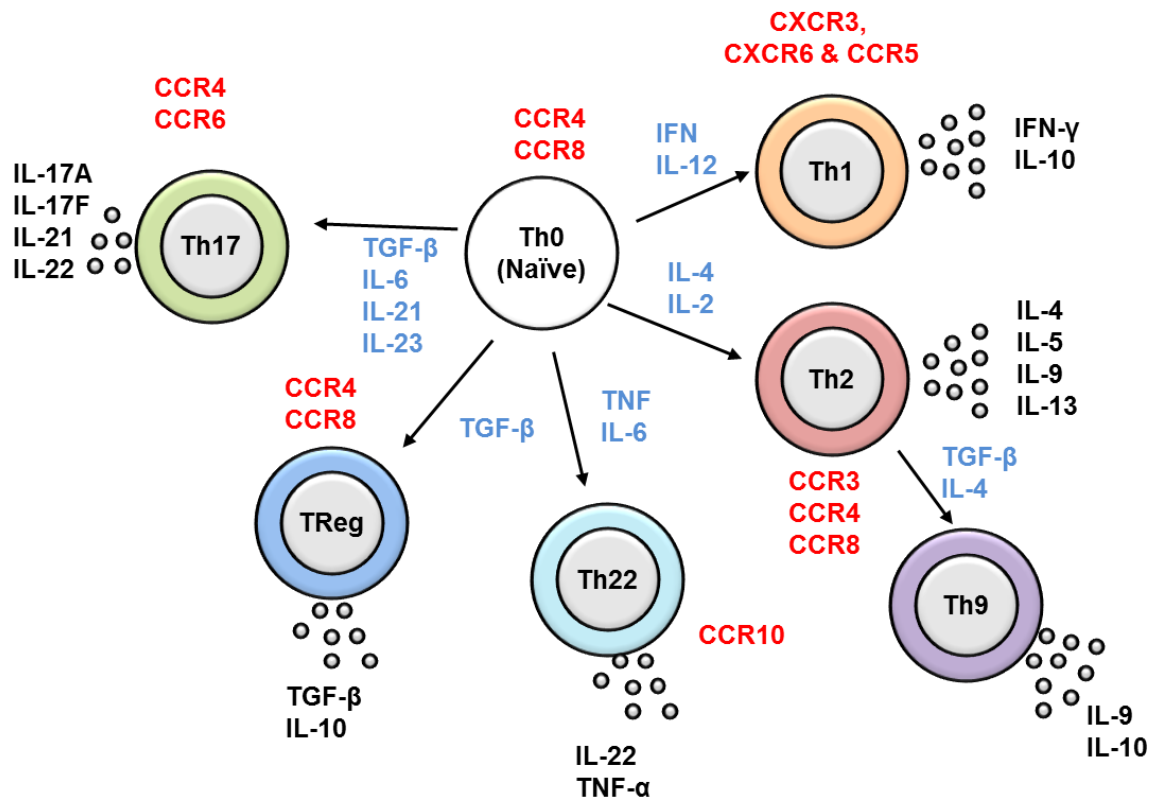


Figure 1.5 Differentiation of CD4⁺ T lymphocytes into distinct subsets. The cytokine environment, indicated in the blue text, surrounding naïve T lymphocytes is able to “skew” them to specific subsets. Typical chemokine receptors associated with specific subsets are stated in red and the cytokines produced by specific subsets are shown in the black text (Original graphic).

1.3 T lymphocyte migration and trafficking

1.3.1 Cell migration and chemotaxis

Migration is vital to T lymphocyte function in all stages of development and activation. Initially, stem cells precursors have to home from the bone marrow to mature in the thymus. Next, naïve T lymphocytes migrate to survey the circulatory and lymphatic systems to maximise their likelihood of antigen detection. When activated, T lymphocytes enter the periphery and are distributed to aid the clearance of pathogens (Figure 1.9). Finally, memory T cells remain distributed throughout the organism and provide localised immuno-surveillance to detect and induce a rapid response to a previously detected antigen. T lymphocytes function locally and their migration is required to be highly co-ordinated. Therefore, understanding the mechanisms which manipulate T lymphocyte migration may lead to the development of treatments of diseases mediated by T lymphocytes. However, cell migration is complex and involves multiple signalling mechanisms which can be distinct depending upon the developmental stage of the T lymphocyte (Marelli-Berg *et al.*, 2008).

The key features which are reported to influence the migration of immune cells include 1) anatomical borders, 2) adhesion to other cells or extracellular matrix, 3) physical and chemical signals which trigger migration and 4) directional cues that guide the cells (Sixt, 2011). Immune cells are able to migrate in the absence of direction cues this is termed chemokinesis (Massberg *et al.*, 2007), however immune cells often undergo co-ordinated and directional migration in response to specific external chemical signals, termed chemotaxis.

Chemotaxis was first described in bacteria by Pfeffer (Pfeffer, 1884) and later in phagocytic leukocytes by Metchnikoff (Metchnikoff, 1893). Since then cell migration has been intensely researched. Co-ordinated migration depends upon the formation of chemoattractant gradients which cells detect and prioritise. Exposure to a chemoattractant gradient leads to small spatial or temporal differences in cell receptor activation that are amplified intracellularly to induce actin polymerisation and protrusion formation at the side of the cell facing the highest concentration of chemoattractant. A polarised morphology is then stabilised by positive-feedback loops at the front of the cell and inhibitory signals in the rest of the cell (Swaney *et al.*, 2010; Insall, 2013; Reig *et al.*, 2014). Thus, receptor activation is described to generate a local asymmetric accumulation of intracellular signalling molecules or “chemotactic compass” toward the highest concentration of chemoattractant.

1.3.2 Chemoattractant gradients

The most frequently investigated chemoattractants are formyl peptides (*i.e* formyl-methionine-leucine-phenylalanine [fMLP]) and chemokines (Section 1.3.4). Size, polarity, diffusion rate and reactivity, as well as the nature of its surrounding environment all affect the ability of a given chemoattractant to form and maintain a stable gradient. Chemoattractants are produced, often in precise spatial locations, and then diffuse to form a gradient which can spread over short or long distances (Majumdar *et al.*, 2014). Gradients can also be maintained by immobilisation of the chemoattractant on tissues by binding to heparin sulphates (Shute, 2012).

Gradients of small highly diffusible molecules are less likely as they are rapidly diffusible and thus would be unable to maintain a gradient long enough to convey signals to migrating cells. However, interestingly hydrogen peroxide (H_2O_2), a highly diffusible molecule, has been shown to form a gradient 100-200 μ m into injured zebrafish tail fin epithelium. The H_2O_2 gradient is maintained by a graded expression of its synthesising enzyme. The injured zebrafish epithelia express higher levels of H_2O_2 producing NADPH oxidase dual oxidase (DUOX) in cells with higher degrees of injury allowing a gradient of H_2O_2 to be produced (Niethammer *et al.*, 2009). The importance of reactive oxygen species and H_2O_2 in immune cell migration is discussed in more detail in Section 1.6.

Most models of cell migration assume that a chemotactic gradient encountered by the cell is at a steady state, static and spatially stable. Moreover, it is often assumed that the gradient is simply created by diffusion of the chemoattractant from the source site towards a target cell (Schneider *et al.*, 2006). However, in a physiological setting there are several other important parameters which could alter the formation and stability of a chemokine gradient. These include the decay of the chemoattractant, which can depend upon the distance of the chemoattractant from a secreting source, sequestration by the extracellular matrix, self-generation resulting in an amplified signal and modification by extracellular enzymes. Therefore in a given environment, cells could be exposed to dynamic and discontinuous gradients (Majumdar *et al.*, 2014). Additionally, cells might detect more than one chemoattractant therefore must be able to prioritise or adapt to multiple distinct cues.

1.3.3 Polarisation and gradient sensing

The ability of a cell to detect the external chemoattractant gradient governs directed cell migration. Remarkably, many cells can detect less than a 5% difference in chemoattractant concentration between the front and back of the cell (Zigmond, 1977; Parent *et al.*, 1999; Brzostowski *et al.*, 2004). Cells will constantly monitor the chemical concentration of chemoattractant over-time through spatial sampling.

Several theories exist to explain how detection of chemoattractant at cell surface receptors, typically GPCRs, leads to the formation of polarised protrusions and directional migration. As described earlier, the “chemotactic compass” model suggests localised accumulation of signalling molecules towards the highest concentrations of chemoattractant. The asymmetric distribution of the product of PI3K signalling PI(3,4,5)P3 at the front of the cell is a hallmark of cell polarisation (Wang *et al.*, 2002a; Wang, 2009). PI3K accumulates at the front of the cell, whereas negative regulator PTEN is found at the rear of the cell. This localised signalling drives the accumulation of downstream small GTPases Rac and Cdc42 to the leading edge of the cell and forms a signalling compass directing the cell towards the chemoattractant gradient. An alternative model describes chemotactic bias, whereby the chemoattractant bias the organisation of protrusions without the need of a signalling compass (Insall, 2010; Insall, 2013).

The main feature of a cell migrating on a 2D surface is the loss of its rounded morphology; instead the cell spreads and develops multiple peripheral protrusions, named lamellae and finger-like filopodia (Ridley *et al.*, 2003). The cell becomes more adherent and one lamellae becomes broader and dominant to become the front of polarised cell (Lamellipodia). The cell moves in the preferred direction by repeating cycles of protrusion of the front region driven by Cdc42 and Rac activation, adhesion to the substrate, detachment of the cell body and rear, and finally the cytoskeleton contraction to pull the cell forward (Sixt, 2011; Sixt *et al.*, 2011; Majumdar *et al.*, 2014). Rho, myosin II and Ca^{2+} -activated proteases work together to alter actomyosin contractility and de-attachment of the cell (Majumdar *et al.*, 2014). *In vivo* imaging in Zebrafish neutrophils indicates that lamellipodia contain highly branched actin filaments containing dynamic F-actin, whereas stable F-actin localises to the back of the cell (Yoo *et al.*, 2010).

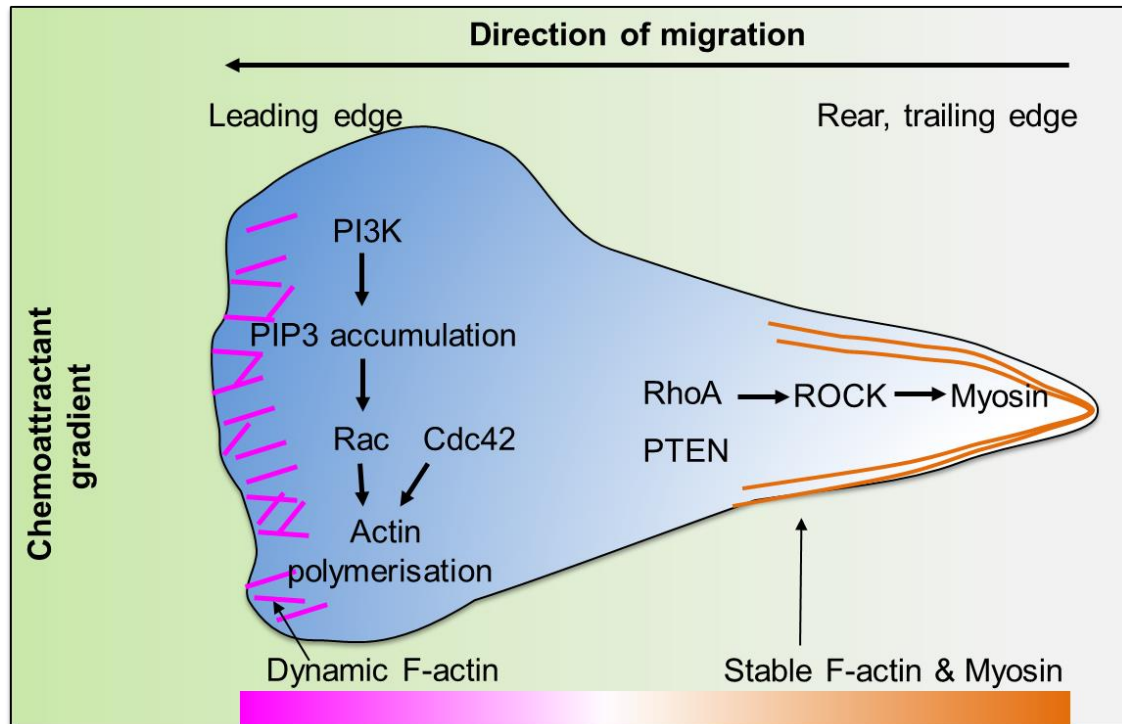


Figure 1.6 Signalling pathways in immune cell polarisation. Upon detection of a chemoattractant gradient, PI3K signalling and PIP3 generation is increased at the leading edge of the cell. This leads to the activation of small GTPases Rac and Cdc42 and subsequent actin polymerisation at the leading edge. At the rear of the cell, phosphatase and tensin homologue (PTEN) prevents PI3K-mediated PIP3 accumulation and myosin contraction is driven by small GTPase RhoA. Adapted from (Deng *et al.*, 2012) and (Yoo *et al.*, 2010).

1.3.4 Chemokines and their receptors

Chemokines are a large family of small 8-10kDa chemoattractant proteins which act to regulate immune cell recruitment (Kim *et al.*, 1999). There are at least 50 known chemokines, however only 20 functional receptors have been described and subgroups of chemokines commonly bind to the same receptor on a target cell. Although chemokines have low sequence identity (Wells *et al.*, 1999), their three dimensional structure exhibits homology (Clark-Lewis *et al.*, 1995) and they are subdivided into four subfamilies based on the position of highly conserved cysteine residues, namely C, CC, CXC and CX3C (Nomenclature 2001; Bacon *et al.*, 2002) which are shown in table 1.1.

The CXC chemokines are further subdivided according to the presence of tri-peptide motif glutamic acid-leucine-arginine (ELR) motif in their NH₂ terminal region. Broadly, ELR+ chemokines are angiogenic and attract neutrophils to the sites of inflammation. Whereas ELR- chemokines, CXCL4, CXCL19, CXCL10, CXCL11-14, and CXCL16 are angiostatic and attract lymphocytes and monocytes (Laing *et al.*, 2004). Chemokines can also be divided by their functional properties; the “homeostatic” are constitutively expressed, whereas the “inflammatory” chemokines are upregulated following inflammatory signals.

Chemokine receptor	Chemokine ligand	Expression
CC family	CCR1	CCL3, CCL5, CCL7, CCL8, CCL14, CCL15, CCL16, CCL23
	CCR2	CCL2, CCL8, CCL7, CCL13, CCL16
	CCR3	CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL24, CCL26, CCL28
	CCR4	CCL17, CCL22
	CCR5	CCL3, CCL4, CCL5, CCL8, CCL13, CCL16
	CCR6	CCL20, CCL21
	CCR7	CCL19, CCL21
	CCR8	CCL1, CCL18
	CCR9	CCL25
	CCR10	CCL27, CCL28
CXC family	CXCR1	CXCL6, CXCL8,
	CXCR2	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8
	CXCR3	CXCL9, CXCL10, CXCL11
	CXCR4	CXCL12
	CXCR5	CXCL13
	CXCR6	CXCL16
	CXCR8	CXCL17
CX3C	CX3CR1	CX3CL1, CCL26
XC	XCR1	XCL1, XCL2
Atypical receptors	ACKR1	CCL2, CCL7, CCL8, CCL13, CCL14, CCL17, CCL22, CXCL1, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL11, CXCL13
	ACKR2	CCL2, CCL3, CXCL1, CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL17, CCL22
	ACKR3	CXCL11, CXCL12
	ACKR4	CCL19, CCL21, CCL25, CXCL13

Table 1.1 The chemokine superfamily. Adapted from (Zlotnik *et al.*, 2012) and (Graham *et al.*, 2012). Chemokine ligands and their receptors are classed into families based on the location of cysteine residues. Atypical chemokine receptors can influence the activity of other chemokines but do not signal through classical pathways. T=T lymphocyte; B = B lymphocyte; Mo = monocyte; Eo = Eosinophil; Ba = Basophil; D= dendritic cell; Tmem = memory T cell; Th = T helper cell; NK = natural killer cell; MC = Mast cell; N= neutrophil, P= platelet, Treg = regulatory T cell, En= endothelial cell, Mac = Macrophage; SMC = smooth muscle cell; HSc = hematopoietic stem cell, VEC = vascular endothelial cells.

Typical chemokine receptors are members of the G-protein coupled receptor family (GPCR), are 340-370 amino acids in length with 25-80% homology (Murphy *et al.*, 2000). GPCRs couple with intracellular heterotrimeric G-proteins, which undertake their down-stream signalling, this is discussed further later in Section 1.4. Chemokines bind to their receptors with nanomolar affinity and binding often is class restricted, for example CXC chemokines only bind to CXC receptors, with the exception of ACKR2 which binds CC and CXC chemokines (Murphy, 1996).

Atypical chemokine receptors are cell surface receptors with seven transmembrane domains that are structurally homologous to GPCRs. However, upon ligation of by their cognate chemokines, atypical chemokine receptors fail to induce classical down-stream signalling. Atypical chemokine receptors can impact on the activity of other chemokine ligands by altering chemokine availability and function (Ulvmar *et al.*, 2011; Graham *et al.*, 2012).

1.3.5 Chemokine receptor internalisation

GPCR signalling can be regulated at the level of receptor expression on the cell surface. Internalisation of the receptor reduces the amount of available GPCR on the cell surface, thus attenuating receptor mediated signalling (Lefkowitz, 1998; Morris *et al.*, 1999). Following ligand activation, most GPCRs are internalised into endosomes and then either targeted for lysosomal degradation or recycled back to the plasma membrane.

There are two main pathways of GPCR internalisation which are (1) arrestin/clathrin pit or the (2) lipid/raft caveolae dependent mechanisms. (1) Briefly, intracellular domains of the receptors are phosphorylated by kinases such as GPCR kinases (GRKs) which decrease the interaction of the GPCR with G proteins and increase their interaction with β -arrestin. β -arrestin binding further inhibits G protein interaction and can activate distinct arrestin-mediated signalling. Furthermore, β -arrestins can directly link with clathrin coated pits to enhance receptor endocytosis (Ferguson, 2001; Luttrell *et al.*, 2002; Kohout *et al.*, 2003; Kohout *et al.*, 2004). (2) It is thought that GPCR signalling components concentrate in membrane micro-domains such as caveolae and lipid rafts in order to undertake rapid signalling, however although it is less well understood that internalisation of receptors in caveolae has been observed and requires dynamin (Chini *et al.*, 2004; Calizo *et al.*, 2012) (Figure 1.7).

A considerable amount of our knowledge about chemokine receptors was initially determined for other GPCRs, including information about receptor trafficking. A few chemokine receptors, including CXCR1, CXCR2, CXCR4 and CCR2 and CCR5, have been more thoroughly investigated and it has been determined that like other GPCRs, chemokine receptor desensitization involves removing them from the plasma membrane by endocytosis. Although trafficking appears to be a conserved trend between chemokine receptors, the mechanisms appear to vary between individual subtypes (Borroni *et al.*, 2010; Bennett *et al.*, 2011).

Surface expression of chemokine receptors can also be altered by gene expression, mRNA stability and protein degradation as well as trafficking. This can lead to both up-regulation and down-regulation of the chemokine receptor (Schioppa *et al.*, 2003; Mehta *et al.*, 2007). Expression of chemokine receptors has to be tightly regulated and their expression profile is altered dependent on an individual cells differentiation, activation and polarisation.

Chemokines receptors can be internalised via clathrin or caveolin-dependent endocytosis, although interestingly other independent pathways have also been reported (Borroni *et al.*, 2010). CCR2 and CCR5 have been shown to follow both clathrin-dependent and caveolae-mediated internalisation pathways and their route of endocytosis could be cell-type dependent (Andjelkovic *et al.*, 2002; Signoret *et al.*, 2005; Garcia Lopez *et al.*, 2009). Sorting of internalised receptor requires complex interactions with intracellular components including PDZ ligand motif and ubiquitination, which support recycling or degradation, respectively. At least 12 chemokine receptors contain the PDZ domain suggesting they are recycled (Borroni *et al.*, 2010). For CXCR4, CXCL12 stimulation appears to lead to ubiquitination and degradation (Marchese *et al.*, 2001; Mines *et al.*, 2009).

Here, the specific interest is CXCR12/CXCR4 homeostatic migration and CXCL11/CXCL10/CXCR3 inflammatory migration of naïve and activated T lymphocytes, respectively. Chemokines and chemokine receptors have been more extensively reviewed here (Olson *et al.*, 2002; Allen *et al.*, 2007; Bennett *et al.*, 2011; Graham *et al.*, 2012).

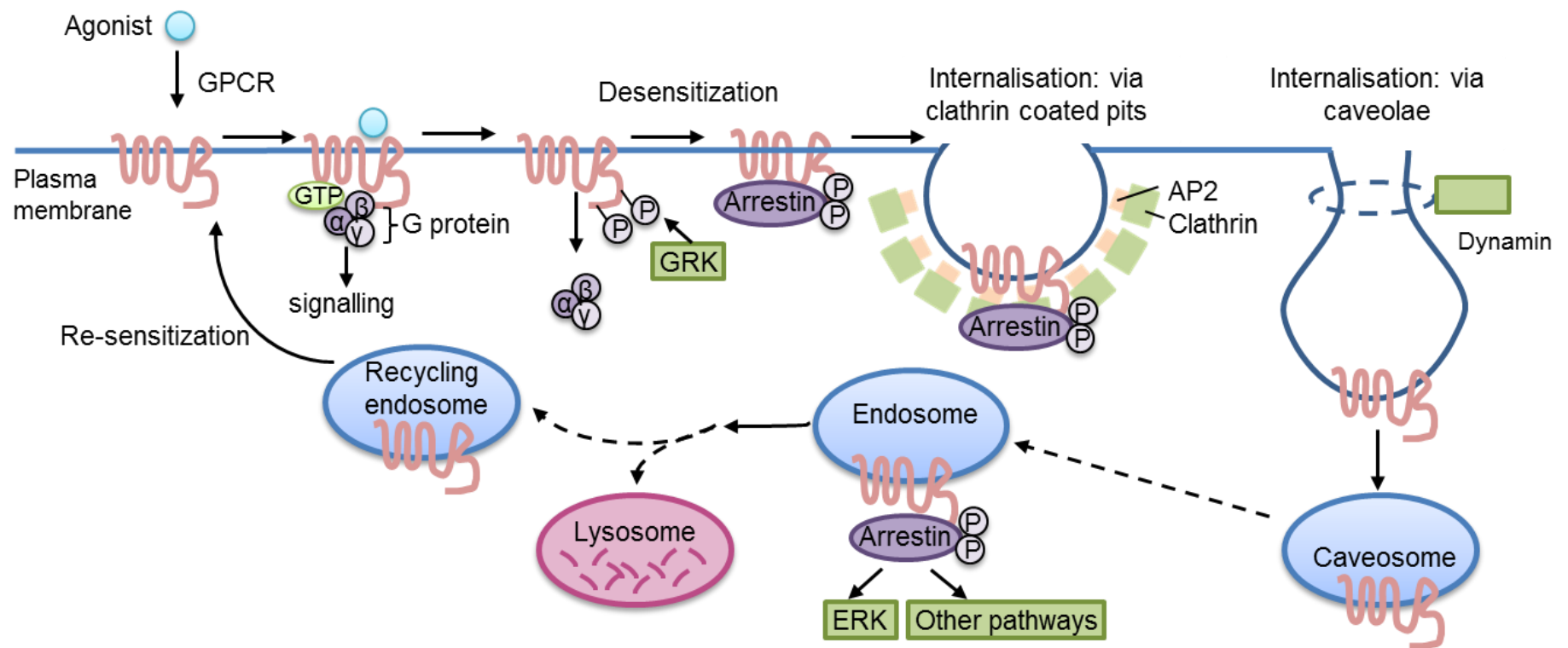


Figure 1.7 Internalisation of GPCRs. G protein-mediated signalling by agonist-activated GPCRs can be terminated through GPCR phosphorylation by GPCR kinases (GRKs) and association with arrestins, which interact with clathrin and the clathrin adaptor AP2 to drive GPCR internalization into endosomes. GPCR internalisation can also occur through dynamin association with caveolae into early endosomes. GPCR internalization regulates the functional processes of receptor desensitization. Recruitment of arrestin to activated GPCRs can also lead to the initiation of distinct arrestin-mediated signalling pathways. Following internalization, GPCRs can be trafficked to lysosomes, where they are ultimately degraded, or to recycling endosomes for recycling back to the cell surface in the functional process of resensitization. Figure adapted from (Ritter *et al.*, 2009).

1.3.6 CXCL12 and the CXCR4 receptor

CXCR4 is one of the best studied chemokine receptors, primarily due to its roles as a co-receptor in HIV entry (Feng *et al.*, 1996) and in cancer metastasis (Zlotnik, 2006). CXCR4 is widely expressed and is present on T lymphocytes, B lymphocytes, monocytes, neutrophils, blood-derived dendritic cells and endothelial cells. CXCR4 can form homo- and heterodimers, with CCR2 and CD4 and possibly CCR5. Mutations which reduce CCR5 surface expression also decrease expression of CXCR4. The functional consequences of CXCR4 heterodimerization are not well understood although it is likely to be an additional means of regulating chemokine signalling (Busillo *et al.*, 2007).

Many of the chemokine receptors are promiscuous and bind several chemokines; however 6 bind a single ligand (Balkwill, 2004). CXCR4 is 352 amino acids and selectively binds the CXC chemokine, CXCL12, also known as stromal development factor 1 (SDF1). CXCR4 binding to CXCL12 initiates survival, growth and chemotaxis pathways. There are currently 6 known splice isoforms of CXCL12 (α , β , γ , δ , ϵ , ϕ) that are comprised of an identical N-terminal core but exhibit varied additions of 1-41 largely basic amino acids at their C-terminal (Yu *et al.*, 2006). These isoforms exhibit different tissue distribution; both α and β isoforms are detected in the primary tumour environment (Orimo *et al.*, 2005) and γ has recently been identified in late stage cancer (Cavnar *et al.*, 2014) however their functional importance is not well understood (Yu *et al.*, 2006).

CXCL12 also binds to atypical chemokine receptor ACKR3, also known as CXCR7, which like other atypical receptors fails to induce classical down-stream signalling. Interestingly, ACKR3 has been shown to interact with β -arrestins and induce G protein-independent signals through ERK-1/2 phosphorylation (Canals *et al.*, 2012). Another hypothesis suggests CXCR7 is a “decoy receptor” or a chemokine scavenger (Rajagopal *et al.*, 2010). However, interestingly dimerization of CXCR4/CXCR7 induces a positive binding co-operatively which enhances CXCR4 signalling mediated by CXCL12 (Sierro *et al.*, 2007).

The CXCR4 has been indicated to be important in the correct formation of the vascular, nervous, hematopoietic and cardiac systems and mice that lack either CXCR4 or CXCL12 exhibit late gestational lethality (Nagasawa *et al.*, 1996; Zou *et al.*, 1998). In the adult, CXCR4 is vital in directional migration of haematopoietic cells. CXCL12 is able to interact with glycosaminoglycans, such as heparin sulphate, and is likely to be immobilised *in vivo* allowing for gradient formation (Hoogewerf *et al.*, 1997). Like

CXCR3, CXCR4 is coupled to G α i, and is able to inhibit adenylyl cyclase, activate Src family tyrosine kinases (SFKs), with the liberated $\beta\gamma$ subunit activating phospholipase C- β and PI3K ultimately leading to gene transcription, cell migration and cell adhesion.

Incorporation of CXCR4 into lipid rafts has been observed, and this could underlie its desensitisation. CXCL12 also stimulates the incorporation of SFKs, focal adhesion kinase, PI3K and the small G protein Rac into lipid rafts, which suggests clustering in lipid rafts is involved in allowing signalling to occur (Wysoczynski *et al.*, 2005). CXCR4 signalling is regulated by de-sensitisation, internalisation and degradation. CXCL12 signalling induces activation the G-protein coupled receptor kinase (GRK) phosphorylates of the serine/threonine residues of the third intracellular loop and the cytoplasmic tail of CXCR4. Phosphorylation allows for the binding of arrestin-2 and or arrestin-3, which successfully uncouple the G-protein and target the receptor for internalisation (Krupnick *et al.*, 1998).

1.3.7 CXCR3 receptor

CXCR3 and its ligands co-ordinate cell mediated immunity in the periphery. CXCR3 is highly expressed on effector T lymphocytes and plays an important role in CD8+ and Th1 cell trafficking and function. CXCR3 is also expressed by a number of other cells including natural killer cells, malignant B lymphocytes, endothelial cells and thymocytes (Thomas *et al.*, 2003).

CXCR3 chemokines, CXCL9 (also known as monokine induced by interferon γ) CXCL10 (interferon-inducible protein-10) and CXCL11 (interferon-inducible T cell α or ITAC) are not detectable within the non-lymphoid tissues under physiological conditions, but are strongly induced by cytokines, particularly interferon γ , during inflammation. High surface expression of CXCR3 is found on activated and memory T lymphocyte and on NK cells and is driven by interferon γ (Cole *et al.*, 1998). CXCL9, CXCL10 and CXCL11 exhibit differences in their kinetics, affinity, tissue expression pattern and their binding/activation requires two distinct intracellular domains of CXCR3. Both CXCL10 and CXCL11 have been implicated as allosteric ligands of CXCR3 (Nedjai *et al.*, 2012) In humans, there is further complexity as there are two splice variants of the CXCR3 receptor, CXCR3-A and CXCR3-B, which exhibit different binding profiles for the ligands. Splice variants of CXCR3 are not observed in rodents which could indicate differences in function of the CXCR3 receptor between humans and rodents (Kelsen *et al.*, 2004).

Like other GPCRs, CXCR3 signalling is regulated by the level of receptor expression at the surface membrane. CXCR3 does not appear to require clathrin-coated pits to internalise the CXCR3 receptor and it has been suggested that CXCR3 is degraded in the endosome following internalisation. It has to be replenished on the membrane by *de novo* synthesis; even 3 hours after treatment with CXCL11 only 70-80% expression of CXCR3 is re-established (Meiser *et al.*, 2008). The regulation of CXCR3 surface expression is very important as CXCR3 has been indicated in playing an important role in the induction and perpetuation of several human inflammatory disorders including atherosclerosis (Mach *et al.*, 1999), autoimmune diseases (Sorensen *et al.*, 1999), transplant rejection (Hancock *et al.*, 2000) and viral infection (Liu *et al.*, 2000). Development of agents to reduce CXCR3-ligand interactions may provide novel treatments of these diseases.

1.3.8 Signals that co-ordinate T lymphocyte migration

(a) Naïve T lymphocyte extravasation

Naive T lymphocytes must overcome shear forces to be able to leave the blood. To do this they form “tethering” contacts with specialised receptors expressed in high endothelial venules (HEVs). HEV are specialized post-capillary venous swellings that have a distinctive cuboidal appearance and enable circulating lymphocytes to directly enter a lymph node from the blood. Lymphocytes in the blood express L-selectin and $\alpha 4$ integrins (e.g VLA-4) on the tips of their microvilli. These form initial tethers to their ligands (e.g. vascular cell adhesion molecule (VCAM-1) on the endothelial cell membranes. After initial tethering, lymphocytes roll on the HEVs using a short-term interaction between CD62L (L-selectin) expressed on the T lymphocytes and addressins present upon the HEVs (Gallatin *et al.*, 1983; Rosen, 2004). Tethering allows T lymphocyte proximity with chemokines (i.e. CCL21) associated with the endothelium. Positively charged, CCL21 is anchored onto the endothelium by binding to negatively charged heparin glycosaminoglycans, this prevents it being washed away by the shear blood flow (Bao *et al.*, 2010). Chemokine-induced signalling activates lymphocyte function associated antigen 1 (LFA1) to bind intercellular adhesion molecule (ICAM-1) present on the T lymphocyte. This association causes T lymphocyte arrest upon the surface of the HEVs followed by the extravasation of T lymphocyte into the T cell zone of lymph nodes (von Andrian *et al.*, 2000; von Andrian *et al.*, 2003) [Figure 1.8].

(b) Retention in the lymph node

Lymph nodes provide an environment which maximises cell to cell collaboration between a T lymphocyte and APCs. It is estimated that a single dendritic cell in the lymph node encounters between 500-5000 T lymphocytes per hour (Bousso *et al.*, 2003; Miller *et al.*, 2004).

After T lymphocytes enter the lymph node their migration within the T cell zone is CCR7-dependent. CCR7-dependent chemokines CCL21 and CCL19 increase T lymphocyte motility and antigen scanning (Kaiser *et al.*, 2005). Competing signals are likely to dictate the longevity of T lymphocytes within the lymph node. Over time, CCL19 induces desensitisation of the CCR7 receptor, which limits the signalling retaining the cells in the lymph node (Bardi *et al.*, 2001; Kohout *et al.*, 2004).

Conversely, sphingosine-1-phosphate (S1P) is involved in the egress of T lymphocytes out of the lymph node. S1P is abundantly expressed in the blood and lymph, but is not present in the T cell zone of the lymph node. Lymphocytes which have recently left the blood have been exposed to high levels of S1P, which induces a transient desensitization of the S1P receptor and therefore these cells remain in the T cell zone to scavenge for antigen (Lo *et al.*, 2005; Arnon *et al.*, 2011). However, overtime the sensitivity of the S1P receptor is restored, and cells egress via the lymphatics (Figure 1.8).

(c) Inflammatory recruitment

Under inflammatory conditions, cytokines are produced within the inflamed tissues and then drained into lymph nodes. This leads to structural changes within the lymph node to further enhance antigen screening by naïve T lymphocytes. Up-regulation of ICAM-1 and CCL21 expression and increased HEV diameter enhance the rate of T lymphocyte entry into the lymph node (Janatpour *et al.*, 2001; Palframan *et al.*, 2001). Once activated, expression of specific chemokine receptors facilitates cell migration to specific sites in the periphery. For example CCR9 initiates homing to the small intestine and E-selectin, CCR4, CCR10 direct migration towards the skin (Picker *et al.*, 1993; Salmi *et al.*, 2005) (Figure 1.8). Moreover, the distinct T helper subsets express characteristic chemokine receptors, shown in Figure 1.5. The signature cytokines released by the T lymphocytes encourage tissue resident cells to release the appropriate chemokines which aids the recruitment of more cells of the same T helper subtype and contributes to a positive feedback loop (Mikhak *et al.*, 2006).

Cytotoxic CD8+ T lymphocytes have critical role in destroying resistant pathogens and malignant cells, the inflammatory environment increases their expression of CCR5. CCR5 detects CCL3, CCL4 and CCL5 gradients, which often emanate from sites where helper CD4+ are in contact with APC allowing cytotoxic cells to aid clearance (Castellino *et al.*, 2006). Once effector immune cells have combated the stimulus that originally activated and recruited them the cells are neglected, die by apoptosis and are then are cleared by macrophages resulting in the resolution of the inflammatory response. However, a few of these cells are retained as memory cells and upon recognition of the same antigen the memory T lymphocytes are rapidly re-activated.

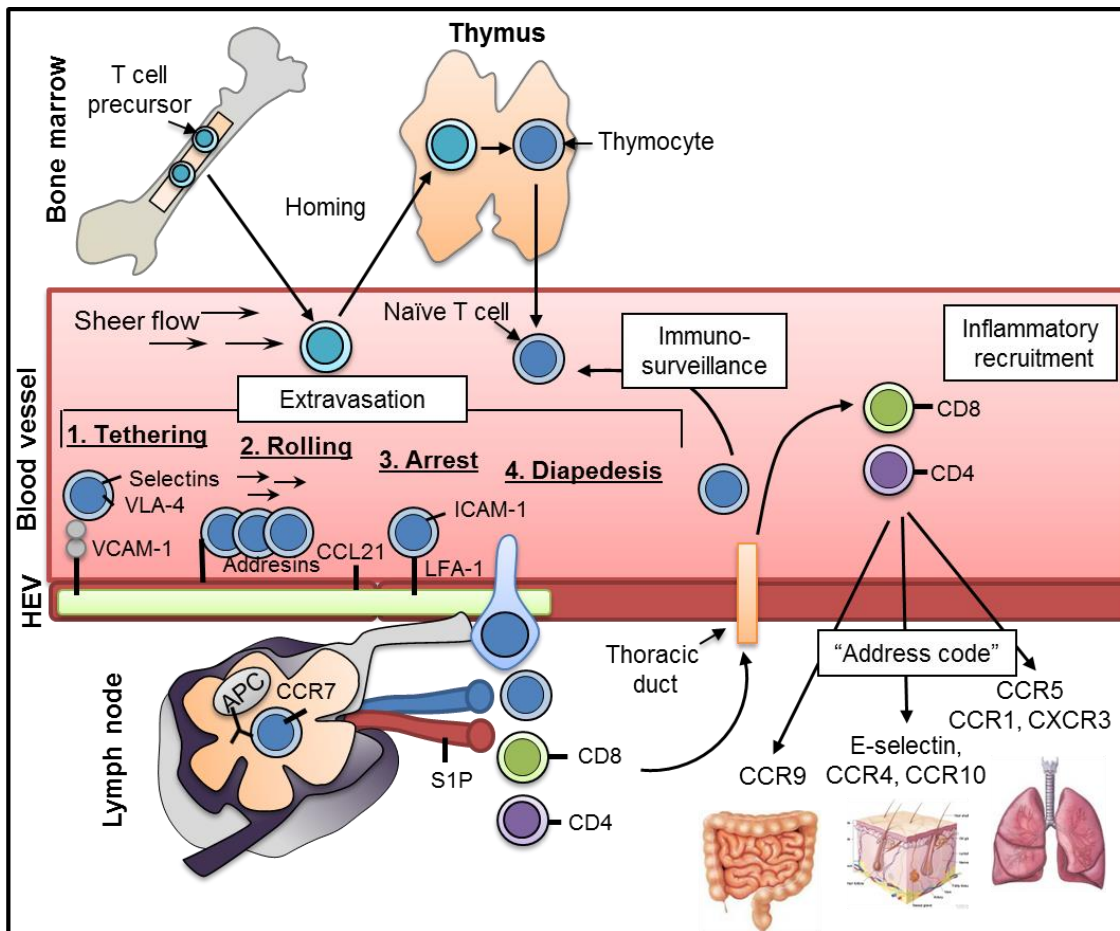


Figure 1.8 T lymphocyte migration. Haematopoietic stem cells from the bone marrow are disseminated into the bloodstream as T cell precursor cells. These cells home to the thymus, where they differentiate into T cells. The T cells are then exported to the periphery, where they recirculate from the blood, through T cell areas of the lymph nodes and back to the blood via the thoracic duct. To be able to leave the blood, under conditions of shear blood flow, T lymphocytes undergo extravasation. **1)** Selectins and VLA-4 expressed on T lymphocytes bind CD62L and VCAM-1 on the endothelial cells, respectively. This forms an initial tethering link between the T cell and the endothelial cell. **2)** Next, T cells begin to roll across the surface of the endothelial cells. **3)** This exposes the T cell to further selectins, addressins and chemokines which evokes the up-regulation of integrins, LFA-1 and VLA-4. These interact with endothelial ligands ICAM-1 and VCAM-1, respectively, to arrest the cell on the endothelium. **4)** Intracellular signalling mechanisms facilitate the spreading, crawling and finally the transmigration of the cells, which can either occur at trans- or para- cellular routes. The transfer of T cell between the blood and lymph nodes often occurs at high endothelial venules (HEV). Under inflammatory conditions, T cells can encounter their specific antigen in the lymph nodes they become activated and expression of specific chemokine receptors facilitates cell migration to specific sites in the periphery, *i.e.* they gain a specific “address code” to migrate to the area of inflammation (Original graphic).

1.4 Signalling pathways involved in T lymphocyte function

T lymphocytes rely upon complex intracellular signal transduction pathways to undertake their functions of migration, activation and cytokine production. In addition, intracellular signalling provides the cell with continuous information about its environment and conveys a large amount of complex information to the cell. Multiple signalling pathways are involved in these processes; however tyrosine kinases, phospholipase C (PLC) and protein kinase C (PKC), calcium mobilisation and phosphoinositide 3-kinase (PI3K) are heavily involved in T lymphocyte responses.

1.4.1 Non receptor, cytoplasmic, tyrosine kinases

There are two major families of cytoplasmic tyrosine kinase families in T lymphocytes namely, the src family kinases (SFK) family and the spleen tyrosine kinases (SYK) family. The kinases are involved in cellular processes such as proliferation, survival, growth, adhesion and migration.

In T lymphocytes, CD3 and CD28 co-receptors contain the immunoreceptor tyrosine-based activation motifs (ITAMs) sequence, YxxLx₍₇₋₁₂₎YxxL, in their intracellular tails. Upon ligation of the TCR receptor, this sequence becomes phosphorylated by Src family kinases (SFKs), Lck and Fyn. Phosphorylated ITAMs recruit Syk kinase family member, ZAP-70 which is tyrosine phosphorylated and activates downstream signalling SLP76 and LAT; which act as linkers to recruit a complex of signalling molecules including VAV1, ITK, GRB2, SOS and PLC γ . In addition, GPCR receptor signalling is able to activate downstream SFK and Syk signalling (Figure 1.9).

(a) SFKs

SFKs are critical in cellular responses to external stimuli and they are activated by a diverse number of factors including cytokines, antigens, integrins and chemokines. SFKs phosphorylate tyrosine residues on target proteins including Tec family member, ITK, SYKs and tyrosine kinase FAK, which activate down-stream signalling including PI3K, Rho/WASP activation of actin polymerisation and PLC/PKC (Thomas *et al.*, 1997).

Conventional Src (c-Src) kinase was the first proto-oncogene discovered encoding a tyrosine kinase and subsequent studies of its function have increased our understanding of the processes involved in malignant transformation (Blume-Jensen *et al.*, 2001). SFKs are a family of 8 non-receptor tyrosine kinases that are divided into two subfamilies, those that are c-Src related c-Src, Yes, Fyn and Fgr and those that are Lyn related Lyn, Hck, Lck and Blk, there are also three related kinases Brk, Frk and Srm (Thomas *et al.*, 1997).

All SFK family members share a similar domain arrangement, including four Src homology domains (SH). Starting at the NH₂-terminal domain, there is a SH4 domain and a “unique” domain, which is highly divergent between members but contains a myristoylation site which allows the SFK to be targeted to the membrane and determines cellular localisation. The SH3 domain detects proline rich motifs which are present in signalling and structural molecules and allows the SFK to be in close proximity to signalling targets. The SH2 domain interacts with phosphotyrosine residues, when SFK are in an inactive conformation the SH2 domain interacts with a phosphorylated tyrosine in its own C-terminal at site 527. This results in a closed, inactive conformation. Finally the SH1 domain is responsible for the enzymatic activity of Src, and auto-phosphorylation at Tyr416 in the SH1 domain results in an open active conformation.

Thus the activity of SFKs is regulated by the phosphorylation and dephosphorylation of tyrosine residues within its own structure. C-terminal Src kinase (Csk) and Csk homology kinase (Chk) are tyrosine kinases which can also inactivate SFK signalling by phosphorylating Tyr527, in particular Chk forms a non-covalent bond with Tyr527 to block kinase activity (Chong *et al.*, 2006; Okada, 2012). Phosphatases including phosphatase 1B and phosphatase- α are capable of removing the phosphate group exposing the catalytic SH1 site (Xu *et al.*, 1999).

SFK expression differs amongst cell types and T lymphocytes predominately express Lck and Fyn which are 56 and 59kDa proteins respectively. Lck is expressed throughout the developmental lifetime of a T cell, whereas Fyn protein is transiently decreased at the double positive stage of thymocyte development but is upregulated thereafter in more mature cells (Cooke *et al.*, 1989; Olszowy *et al.*, 1995). Lck and Fyn have distinct localization within T cells. Lck has been shown to associate with the cytoplasmic tails of T-cell co-receptors CD4 and CD8 (Rudd *et al.*, 1988; Shaw *et al.*, 1989) and commonly directs signalling through ZAP-70 to co-ordinate cell activation and survival. Fyn has been observed to be largely co-localised with the centrosomal and mitotic structures (Ley *et al.*, 1994). Fyn can also interact with the focal adhesion kinase (FAK) family (Qian *et al.*, 1997) and also activates WASP in T lymphocyte (Badour *et al.*, 2004) indicating a role for Fyn in migration, adhesion and synapse formation.

SFK signalling can also be activated downstream of GPCRs, G-proteins been shown to directly stimulate c-Src and Hck, by evoking a conformational change in their structure to reveal their active sites (Ma *et al.*, 2000). Lyn kinase activation has also been observed downstream of CXCR4 and CCR5 signalling in macrophages.

(b) SYK

There are two members of the splenic tyrosine kinase family (SYK) kinases, namely Syk and Zeta-chain associated kinase 70kDa (ZAP-70). SYK are non-receptor, cytoplasmic, tyrosine kinases that have been implicated in the activation of PI3K, PLC γ , MAPK, nuclear factor- κ B and the mobilization of calcium from intracellular stores (Rao *et al.*, 1997; Lewis, 2001). SYKs share a characteristic dual SH2 domain separated by a linker domain (Au-Yeung *et al.*, 2009).

Typically, SFKs phosphorylate the ITAM adaptors which create docking sites for SYK kinases, thus SYK signalling is dependent upon SFK signalling. Interestingly, genetic silencing and pharmacological blockade studies have indicated that inhibition of SFKs and SYK does not always produce the same phenotypic consequences or effect downstream signalling in the same way. Instead, there is a high level of redundancy between the different SFK isoforms and they are involved in signalling pathways which do not involve SYK activation. As such, SFKs have been described to tune signalling responses in a graded fashion and although SYK signalling is also involved in broad

functional effects they are more defined due to a lack of redundancy and are critical in certain pathways (Lowell, 2011).

Syk is abundant in B lymphocytes and expressed at lower levels in immature T cells, mast cells, endothelial cells and platelets. Syk has key roles in B lymphocyte proliferation and migration and in developmental angiogenesis (Turner *et al.*, 2000). In particular, it has recently been indicated that Syk has a key role in co-ordinating B lymphocyte actin cytoskeleton re-organisation, Fc γ receptor clustering (Jaumouille *et al.*, 2014) and Syk signalling is up-regulated in acute B lymphoblastic leukaemia (Uckun *et al.*, 2014)

In contrast, ZAP-70 is predominately expressed in T lymphocytes and has an imperative role in their activation and development. ZAP-70 deficiency mutations in human T lymphocytes causes patient's to exhibit severe combined immune deficiency syndrome (Elder *et al.*, 1994; Matsuda *et al.*, 1999). ZAP-70 is known to associate with phosphorylated ITAMs in the CD3 and ζ chain dimers of the TCR receptor and mediate signalling. In addition, ZAP-70 has also been shown to be required for CXCR4 mediated migration of T lymphocytes (Ticchioni *et al.*, 2002) in the absence of a functional TCR receptor. This implies that ZAP-70 is activated downstream of CXCR4 ligation; however the requirement of SYK induced signalling in migration is less well understood than in TCR activation.

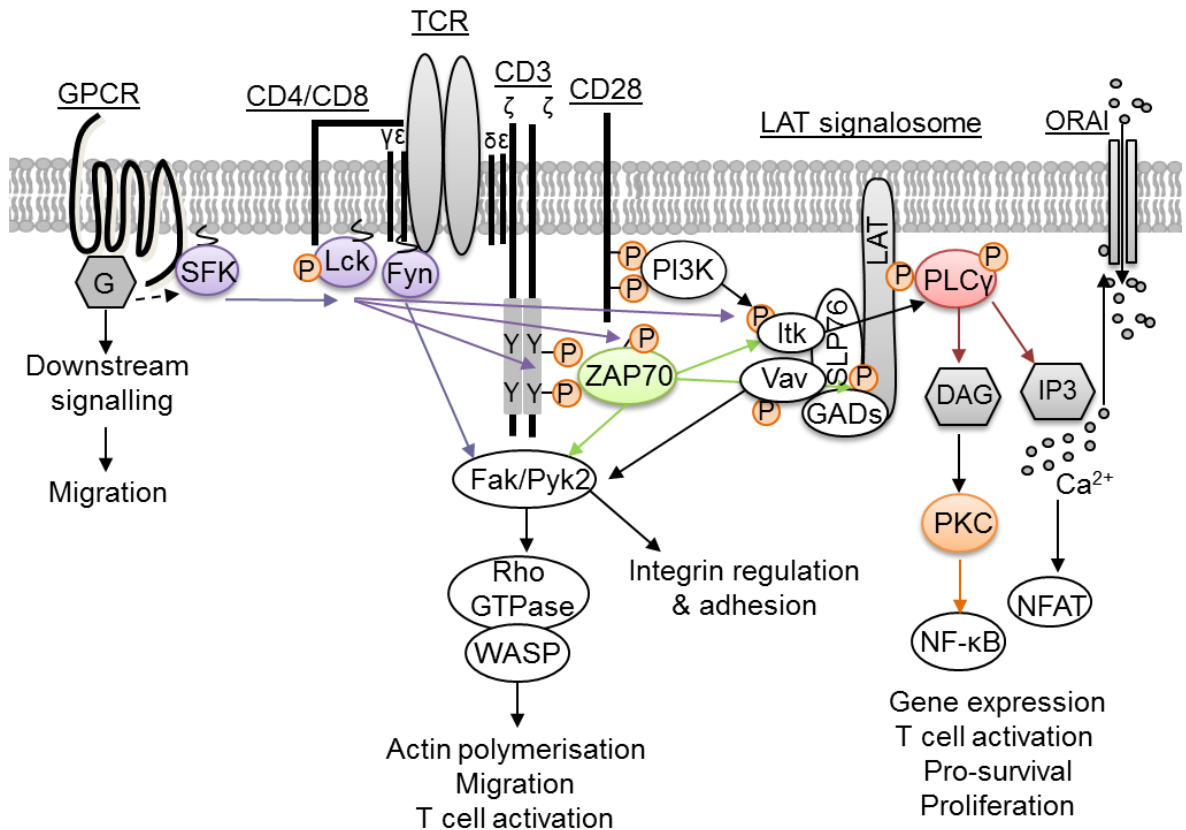


Figure 1.9 SFK and SYK signalling in T lymphocytes. SFK and SYK signalling is activated downstream of TCR and GPCR activation. Following cell activation, SFKs typically phosphorylate ITAM containing adaptors, CD3, CD28 to create docking sites for SYK kinases. SFKs also phosphorylate ZAP-70, Tec kinase Itk and Fak/Pyk2 (Shown in purple) to activate signalling involved in T cell activation and migration.. ZAP-70 is activated by the SFK tyrosine phosphorylation and is recruited to the phosphorylated ITAM domains. Next ZAP-70 phosphorylates SLP76 and LAT (Shown in green) and co-ordinates the formation of the LAT signalosome. PLCγ1 is recruited and hydrolyses its substrate PI(4,5)P2 into IP3 and diacylglycerol (DAG). IP3 acts upon IP3 receptors in the endoplasmic reticulum and stimulates the release of calcium (Ca²⁺). Intracellular Ca²⁺ evokes Ca²⁺-dependent Ca²⁺ influx, through STIM and ORAI channels in the plasma membrane to enhance the Ca²⁺ signal. Ca²⁺ activates transcription factor Nuclear Factor of Activated T lymphocytes (NFAT) leading to synthesis of IL-2. DAG activates PKC signalling and the Ras/MAPK pathway to regulate gene expression and T lymphocyte activation. ZAP-70 also activates Fak/Pyk2 which initiates signalling to regulate focal adhesions and cell movement (Own graphic).

1.4.2 Pharmacological targeting of SFKs and SYK

Given the importance of SYK signalling downstream of ITAM domains, its inhibition has therapeutic potential to treat a number of immune related diseases. Abnormal signalling through SYK has been implicated in several hematopoietic malignancies (Calpe *et al.*, 2013; Tan *et al.*, 2013; Puissant *et al.*, 2014; Krisenko *et al.*, 2015). Several transforming viruses contain ITAM domains including Epstein Barr virus, which lead to the pathological activation of SYK. Thus, Syk inhibition has been proposed as a therapy for lymphoma and chronic lymphocytic and inhibitors are in clinical trials (Friedberg *et al.*, 2010). Syk inhibition is also putative treatment of auto-immune diseases. Fostamatinib (R788) an orally active pan inhibitor has recently produced promising results in a phase II clinical trial for human rheumatoid arthritis which is resistant to methotrexate (Kaur *et al.*, 2014).

Novel pan inhibitors of SFKs have also been designed and are being investigated in the treatment of cancer. Dasatinib (BMS-354825), a selective pan SFK inhibitor is currently approved for the second line treatment of chronic myeloid leukaemia and several other inhibitors are in clinical trials for the treatment of solid tumours. Dasatinib also has a potential therapeutic use in prostate cancer (Nam *et al.*, 2005) and inhibits T lymphocyte activation and proliferation so could be useful in autoimmune disease and transplant allograft rejection (Schade *et al.*, 2008), although long term inhibition of SFK could result in several side effects due to its wide spread functions.

1.4.3 PLC, PKC and calcium mobilisation

PLC and PKC have been demonstrated to be activated downstream of chemokine receptors and TCR ligation. TCR activation evokes PLC γ recruitment (Irvin *et al.*, 2000) and chemokine receptor activation leads to the activation of PLC β either directly or via signalling of several protein kinases (Wu *et al.*, 1993). The PLC family contains 6 isoforms, namely β , γ , δ , and the more recently identified ϵ , ζ , η (Katan, 1996; Hwang *et al.*, 2005).

PLC recruitment and activation brings it into close proximity with its membrane anchored substrate PI[4,5]P₂ and results in its hydrolysis into two second messengers diacylglycerol (DAG) and inositol triphosphate (IP₃). IP₃ binds to the IP₃ receptor (IP₃R), which is itself a calcium permeable ion channel, and evokes the release of Ca²⁺ from intracellular endoplasmic reticulum (ER) Ca²⁺ stores. Ca²⁺ release from the ER activates STIM or ORAI channels in the plasma member. Intracellular Ca²⁺ concentrations are crucial for the activation of gene transcription and cytokine gene expression, as well as cytoskeleton rearrangements (Lewis, 2001; Feske, 2007). PLC β are the most abundantly expressed in the immune system and are involved in neutrophil function (Tang *et al.*, 2011).

DAG is highly hydrophilic, and anchors to both conventional PKC (cPKC - α , β and γ) and novel PKC (nPKC - δ , ϵ , η and θ) isoforms at the inner leaflet. There are three groups of PKC isoforms, cPKC are sensitive to Ca²⁺ and DAG binding, whereas nPKC are DAG sensitive but Ca²⁺ insensitive and atypical PKCs ζ and λ contain altered C1 domains and are both DAG and calcium insensitive. nPKCs (apart from δ) have been shown to be required for microtubule organising centre (MTOC) to the immunological synapse (Quann *et al.*, 2009; Quann *et al.*, 2011). PKC θ alongside GRB2 and SOS activate of GTPase Ras which has multiple roles in cell growth (Roose *et al.*, 2005). In addition, other PKC substrates include PKD1 which forms a complex with adhesion and degranulation promoting adaptor protein (ADAP) and SLP76 and RAPL which are involved in integrin activation (Trucy *et al.*, 2006). Consistently, PKC has also been found to be involved in migration of T cells (Cronshaw *et al.*, 2006).

1.4.4 PI3K family signalling

PI3K activation co-ordinates cell activation, growth, survival, proliferation, differentiation and the homing of inflammatory cells to the site of infection (Courtneidge *et al.*, 1987; Vanhaesebroeck *et al.*, 2012). Four distinct PI3K subclasses exist, which are designated Class I, II, III and IV due to their subunit composition, substrate specificity and regulation. Functionally, Class I PI3Ks phosphorylate the 3' hydroxyl group of inositol ring of phosphoinositol (4,5) bis-phosphate (PI(4,5)P₂) to convert it to phosphoinositol (3,4,5) tris-phosphate (PI(3,4,5)P₃), whereas Class II PI3Ks convert PI-4-P into PI-3,4-P₂ and the conversion of PI to PI-3-P can be mediated by both Class II and III PI3Ks. Class I PI3Ks are the most extensively studied, and their product PI(3,4,5)P₃ is able to recruit and co-ordinate the activation of pleckstrin homology (PH) domain containing proteins, of which the most notable is the serine threonine protein kinase B (Akt). Akt activation initiates a plethora of signalling cascades which are described in detail in Figure 1.10.

Class I PI3Ks are heterodimers comprised of a 110kDa catalytic subunit and a smaller Src Homology 2 (SH2) domain containing regulatory subunit. PI3K regulatory subunits have two vital functions in quiescent cells which are to inhibit PI3Ks functional activity and prevent the catalytic subunit protosomal degradation (Yu *et al.*, 1998). Class I PI3Ks are further subdivided into two classes; the Class IA isoforms are composed of the catalytic subunits p110 α , p110 β and p110 δ , which are associated with the regulatory subunits p85 α , p55 α , p50 α , p85 β or p55 γ , whereas the Class IB catalytic subunit is p110 γ which associates with either p84/p87. Class IA PI3K are principally activated by receptor tyrosine kinases (*i.e.* cytokine and antigen receptors), as their regulatory subunits contain SH2 domains which allow their recruitment to tyrosine phosphorylated proteins at the plasma membrane. In contrast, Class IB PI3Ks are generally activated upon G-protein coupled receptor (GPCR) ligation (*i.e.* chemokine receptors) (Foster *et al.*, 2012; Vanhaesebroeck *et al.*, 2012) However, PI3K β can also couple to GPCRs (Guillermet-Guibert *et al.*, 2008).

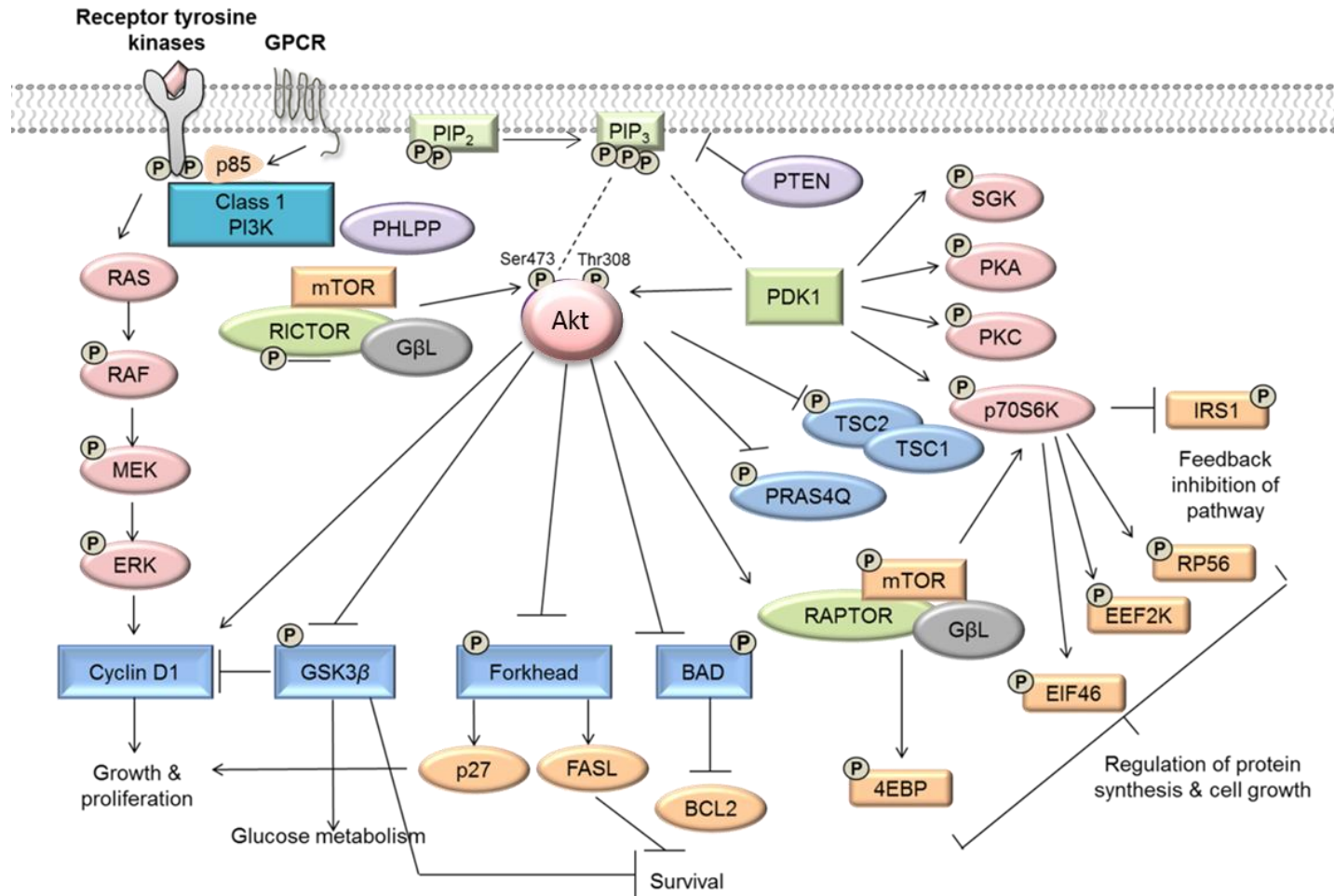


Figure 1.10 Class I PI3K signalling pathways involved in regulating cell function. Adapted from (Workman *et al.*, 2012), this diagram indicates the core components of PI3K signalling. Abbreviations include glycogen synthase kinase-3 (GSK-3 β), Bcl-2-associated death promoter (BAD), 3-phosphoinositide-dependent kinase-1 (PDK-1), phospholipase C (PLC), protein kinase A (PKA), mammalian target of rapamycin complex (mTOR), Phosphatase and tensin homolog (PTEN), Serine/threonine-protein kinase (SGK), Tuberous sclerosis protein 1 & 2 (TSC1; TSC2) Ribosomal protein S6 kinase beta-1 (p70S6K) extracellular-signal-regulated kinases (ERK1/2), mitogen-activated protein kinase kinase (MEK), Fas ligand (FASL), B-cell lymphoma 2 (BCL2), Eukaryotic translation initiation factor 4E-binding protein 1 (4EBP), Eukaryotic translation initiation factor 4E (EIF46) and Eukaryotic elongation factor-2 kinase (EEF2K).

1.4.5 Pharmacological targeting of PI3K

Activation of PI3K initiates signalling cascades involved in the regulation of numerous cellular functions, such as growth, proliferation, migration and cytokine production (Fruman *et al.*, 2009; Sasaki *et al.*, 2009; So *et al.*, 2012a) and is crucial for normal functioning of the immune system. Over-active and dysregulated PI3K signalling is however associated with chronic inflammation, immune-mediated disorders as well as cancer (Yuan *et al.*, 2008; Ghigo *et al.*, 2010; Marwick *et al.*, 2010; Suarez-Fueyo *et al.*, 2011). Specifically, the hematopoietic cell restricted PI3K isoforms (γ and δ) are indicated as eliciting co-ordinated but non-redundant roles in the immune system. As such, null p110 γ and p110 δ mice exhibit severely defective immune responses to infection (Ji *et al.*, 2007). Based on these findings selective inhibition of PI3K δ or PI3K γ isoforms was proposed as a putative treatment of haematological malignancies and autoimmune/inflammatory disease. Targeting these isoforms with their restricted expression profile should prevent widespread inhibition of other more widely expressed isoforms (e.g. p110 α , p110 β). Although there has been success at designing PI3K δ inhibitors which have promising results against hematopoietic cancer, with PI3K δ inhibitor Idelalisib now approved for treatment of CLL in the US (Wong *et al.*, 2010; Foster *et al.*, 2012; Meadows *et al.*, 2012; So *et al.*, 2012a), as yet the use of PI3K inhibitors to treat inflammatory disorders has been disappointing.

A phase II study of PI3K $\gamma\delta$ inhibitor duvelisib (IPI-145) failed to meet its primary endpoint, which led to Infinity Pharmaceuticals announcing that it will not proceed with any further development of PI3K inhibitors in rheumatoid arthritis; however it will continue to evaluate duvelisib for the treatment of hematopoietic cancers. [Infinity press release (Infinity, 2015)]. In addition, Gilead sciences have terminated development of CAL-263 after a Phase I trial for allergic rhinitis, and idelasib (CAL-101) no longer appears upon their development pipeline for the treatment of inflammatory diseases following completion of its Phase I trial for allergic rhinitis (Gilead website). There is the possibility that PI3K γ inhibitors could be useful in inflammatory diseases, however it has been very difficult to design highly selective PI3K γ inhibitors with an acceptable window of selectivity for p110 γ versus other class IA isoforms and this has only recently been achieved (Bergamini *et al.*, 2012). PI3K inhibitors that are currently in clinical trials are summarised in table 1.2 and IC₅₀ values of commonly used pan PI3K inhibitors and selective p110 γ and p110 δ inhibitors shown in Table 1.3

Drug & Company	Target	Therapeutic target	Toxicities	Phase of clinical trial
Pictilisib (GDC-0941) Roche	p110- α , - β - γ , - δ	Breast, NSCLC, melanoma, endometrial, pancreatic tumours	Nausea, Diarrhoea, rash, vomiting	IB/II
Taselisib (GDC-0032) Genentech	Mutated p110- α , PIK3CA	Breast tumours, NSCLC	Diarrhoea, rash, vomiting, anorexia	I
Idelalisib (CAL-101) Gilead	p110- δ	CLL/SLL/iNHL, MCL	Pyrexia, nausea, fatigue	Approved CLL & FL
Buparlisib (BKM120) Novartis	p110- α , - β , - γ , - δ	Advanced breast cancer, Endometrial cancer	Rash, diarrhoea, mood alteration	III
Dactolisib (BEZ235) Novartis	p110- α , - β , - γ , - δ /mTOR	Breast tumours, GBM	Mucositis	IB/II
Copanlisib (BAY80-6946) Bayer	p110- α , - δ	CLL, NHL	Nausea, diarrhoea, anaemia, mucositis & fatigue	II
Duvelisib (IPI-145) Infinity	p110, - γ , - δ	Blood cancers. CLL, MM, iNHL, Asthma	Hepatic toxicity, respiratory infection	Ila - asthma, I - cancer
Omipalisib (GSK-2126458) GlaxoSmithKline	p110- α , - β , - γ , - δ /mTOR	RCC, bladder tumours, IPF	Nausea, vomiting, diarrhoea	I
RP6530 Rhizen	p110, - γ , - δ	B and T cell malignancies & CLL	N/a	Recruiting Phase I
AMG-319 Amgen	p110, - δ	CLL & NHL	Anaemia, nausea	Phase I ongoing
TG-1202 (RP5264) TG/Rhizen	p110, - δ	CLL & NHL	N/a	Phase I (recruiting)
AQX-1125 Aquinox	Activation of SHIP-1	Asthma, COPD, Bladder pain/cystitis	Mild nausea, abdominal pain and dyspepsia	Ila

Table 1.2 PI3K inhibitors and SHIP-1 activator currently in clinical trials. Details for PI3K/mTOR pathway inhibitors that are currently in clinical trials, including their associated companies, their isoform targets, clinical indication, reported side effects and phase of clinical development. SHIP-1 activator is highlighted in pink. First approved in series highlighted green and inhibitors developed for inflammatory disease highlighted blue. Abbreviations: chronic lymphocytic leukaemia, (CLL) small lymphocytic lymphoma (SLL) Indolent non-Hodgkin lymphoma (iNHL) mantle cell lymphoma (MCL) glioblastoma multiforme (GBM) multiple myeloma (MM), renal cell carcinoma (RCC) non-Hodgkin lymphoma (NHL), idiopathic pulmonary fibrosis (IPF). References: Pictilisib (Wallin *et al.*, 2012; Munugalavada *et al.*, 2014), Taselisib (Ndubaku *et al.*, 2013), Idelalisib (Inman, 2014; Okoli *et al.*, 2015), Buparlisib (Bendell *et al.*, 2012) Dactolisib (Chen *et al.*, 2014; Wang *et al.*, 2014) Copanlisib (Cheng *et al.*, 2011; Glauer *et al.*, 2013) IPI-145 (Dong *et al.*, 2014) Omipalisib (Leung *et al.*, 2011; Norman, 2014), RP6530: (Savona *et al.*, 2013), AMG-319 (Castillo *et al.*, 2014; Sinclair A *et al.*, 2011) and TG-1202 (Burris *et al.*, 2014). SHIP-1 activator AQX-1125 (Stenton *et al.*, 2013a; Leaker *et al.*, 2014).

	IC ₅₀ values (nM)				
	PI3K isoforms				
Compound	p110α	p110β	p110δ	p110γ	References
ZSTK474	16	44	4.6	49	(Kong <i>et al.</i> , 2007)
LY294002	5500	1100	160	1200	“
AS-605240	3400	>20,000	>20,000	190	(Bilancio <i>et al.</i> , 2006)
IC87114	>100,000	1820	70	1240	“

Table 1.3 IC₅₀ values of commonly used pan PI3K inhibitors and selective p110 γ and p110 δ inhibitors. Individual IC₅₀ values from *in vitro* assays with either purified or recombinant proteins.

1.4.6 Novel approaches to manipulate PI3K signalling in the immune system

The lack of effective PI3K inhibitors in the treatment of inflammatory pathologies has led to increased interest in other candidates within the PI3K signalling cascade such as the lipid phosphatase SH2 domain containing inositol phosphatase 1 (SHIP-1) which physiologically modulates PI3K-dependent signalling (Blunt *et al.*, 2012a; Blunt *et al.*, 2012b).

PI(3,4,5)P₃ accumulation is counteracted by two intracellular lipid phosphatase families; namely phosphatase and tensin homology (PTEN) and SH-2 domain containing inositol phosphatase (SHIP). Enzymatically, lipid phosphatase PTEN functions to directly inhibit PI3K-dependent signalling by removing the 3' phosphate group of PI(3,4,5)P₃ and converting it back to PI(4,5)P₂ (Figure 1.11). In contrast, SHIP hydrolyses 5' phosphate from PI(3,4,5)P₃ to produce PI(3,4)P₂. Activation of either PTEN or SHIP-1 is proposed to mimic the effect of a pan PI3K inhibitor as they encourage a reduction of cellular PI(3,4,5)P₃ accumulation, however SHIP-1 is a highly attractive target for the treatment of autoimmune and inflammatory diseases and blood cancers due to its primarily restricted expression in hematopoietic cell lineages (Blunt *et al.*, 2012a).

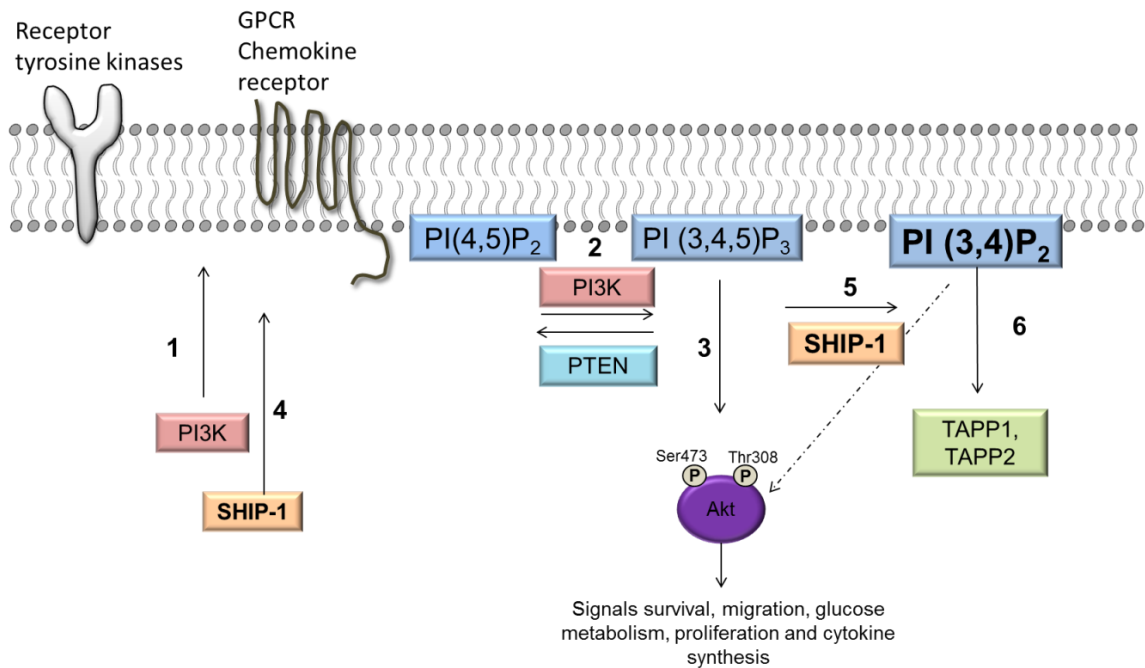


Figure 1.11 Regulation of PI3K signalling. 1. Recruitment and activation of PI3K occurs upon stimulation of receptor tyrosine kinases (i.e. cytokine or antigen receptors), tyrosine coupled-receptors and GPCRs (chemokine receptors). 2. Active PI3K phosphorylates the 3' region of PI(4,5)P₂ to the signalling lipid PI(3,4,5)P₃. 3. Recruitment and phosphorylation of the PH domain containing Akt mediates a plethora of signalling cascades which regulate cell migration, proliferation, survival, glucose metabolism and cytokine synthesis. 4. Ligation of GPCR chemokine receptors and receptor tyrosine kinases/ tyrosine-coupled receptors evokes the relocation of lipid phosphatase SHIP-1 to the plasma membrane. 5. SHIP-1 dephosphorylates the 5' region of PI(3,4,5)P₃ to PI(3,4)P₂ and thus negatively regulates PI3K-dependent signalling. 6. However, PI(3,4)P₂ still has signalling properties and is able to exclusively recruit TAPP1 & TAPP2 which can also negatively regulate PI3K signalling. In addition, PI(3,4)P₂ has a putative role in cell survival pathways and is suggested to be required for full activity of Akt.

1.5 SHIP-1

There are three known isoforms of SHIP; SHIP-1 is restricted to hematopoietic cells, SHIP-2 is widely expressed with particularly high levels in the brain, heart and skeletal muscles and s-SHIP is primarily reported to be restricted to stem cells, their structures are shown in Figure 1.12. In addition to its catalytic role, SHIP-1 is a scaffolding protein which interacts with Growth factor receptor-bound protein (Grb), SH2 domain-containing protein-tyrosine phosphatase (SHP-2), Docking proteins (Doks), GRB2-associated-binding protein (Gab) and immunoreceptor tyrosine-based inhibition/activation motifs (ITIMs and ITAMs). As such, SHIP-1 acts as a pluripotent regulator of hematopoietic cell function (Ming-Lum *et al.*, 2012). Most of these interactions contribute to the negative regulation of PI3K signalling by SHIP independently of its catalytic activity. For example, binding of SHIP-1 to ITAM containing adaptor proteins via the SH2 domain, can prevent PI3K recruitment via the p85 regulatory subunit (Peng *et al.*, 2010) and impaired recruitment of the tyrosine phosphatase SHP1 to the SLAM family receptor 2B4 in NK cells (Wahle *et al.*, 2007). However, there is evidence that the scaffolding role of SHIP-1 can potentiate EGF-induced PLC-gamma1 activation (Song *et al.*, 2005) and facilitates a positive regulatory role in TLR-induced cytokine production (Ruschmann *et al.*, 2010). In addition, a pleckstrin homology (PH)-related (PH-R) domain binds PI(3,4,5)P₃ and is required for localization of SHIP-1 to the phagocytic cup and SHIP-1 mediated inhibition of FcγR-mediated phagocytosis by macrophages (Ming-Lum *et al.*, 2012).

Although SHIP-1 is catalytically active in the cytosol, physical recruitment to the plasma membrane is required for its ability to function upon its substrate PI(3,4,5)P₃. Ligation of chemokine, antigen, co-stimulatory and cytokine receptors as well as IgG engagement by FcγRIIB are all able to recruit SHIP-1 to the plasma membrane (Bruhns *et al.*, 2000) and phosphorylate the C terminal NPXY region of SHIP-1. Compounds which restrict or enhance SHIP-1 recruitment could function as activators or inhibitors of PI3K signalling respectively. Interactions between CD3 and the TCR ζ chain with the SH2 domain of SHIP-1 have been observed *in vitro* (Osborne *et al.*, 1996). In addition, the stimulation of CD3 and CD28 has been shown to cause tyrosine phosphorylation, increased catalytic activity, and a marked re-distribution of SHIP-1 from the cytosol to the surface membrane (Edmunds *et al.*, 1999).

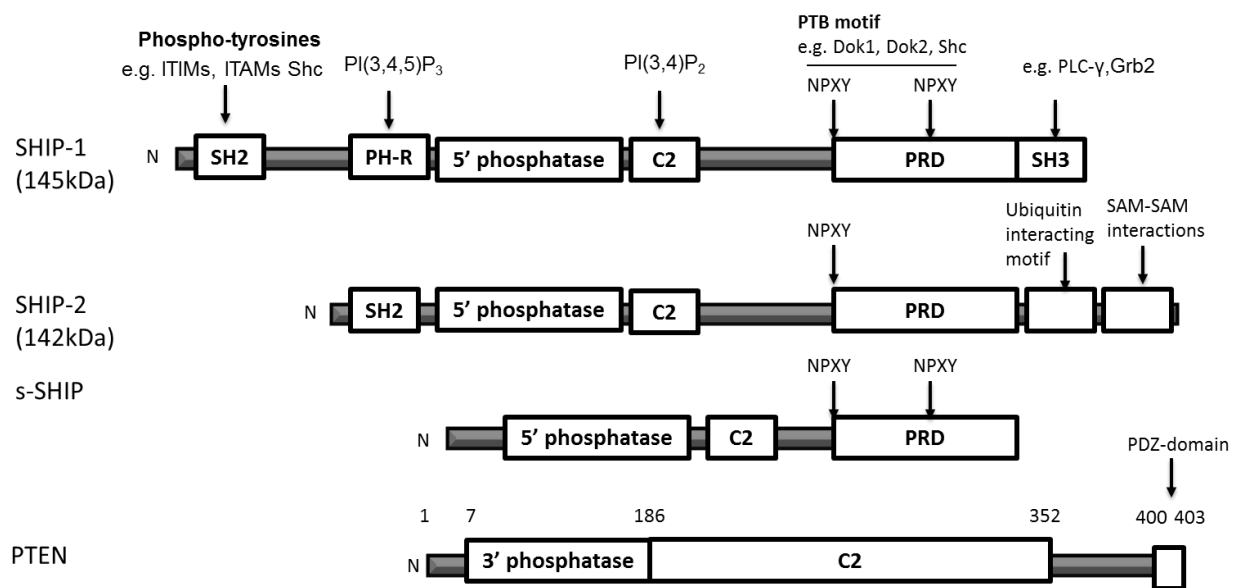


Figure 1.12 Schematic representation of structure of the lipid phosphatase regulators of PI3K mediated signalling. SHIP-1 structure includes a N-terminal SH2 domain, which can bind phospho-tyrosine, and a recently identified PH-related domain, which can associate with PI(3,4,5)P₃. The catalytic phosphatase region of SHIP-1 is centrally located next to a C2 domain which can bind PI(3,4)P₂ and allosterically enhance SHIP-1's catalytic activity. The C-terminal of SHIP-1 has a proline rich domain and NPXY motifs. In contrast to SHIP-1, SHIP-2 also processes a Ubiquitin-interacting motif and a SAM-SAM interaction domain and s-SHIP lacks a SH2 domain. PTEN structure contains a 3' phosphatase region, a C2 region which binds to the phospholipid membrane in order to bring PTEN into close proximity with PI(3,4,5)P₃ and a PDZ domain.

1.5.1 Evidence for the role of SHIP-1 in disease pathogenesis

(a) Cancer

PI3K-dependent signalling presents a compelling target for the treatment of cancers due to known roles in cell survival, proliferation and differentiation (Yuan *et al.*, 2008). In addition, PI3K/AKT/mTOR signalling is consistently over-active in various cancers. It is therefore unsurprising that PTEN is a well characterised tumor suppressor gene (Li *et al.*, 1997; Choi *et al.*, 2002; Sulis *et al.*, 2003; Downes *et al.*, 2007) and this would also imply that hematopoietic restricted SHIP-1 could exhibit a role in blood cancer treatment. Inactive SHIP-1 mutants are present in lymphoblastic and acute myeloid leukaemia (Luo *et al.*, 2003; Luo *et al.*, 2004). Likewise, SHIP-1 is lost in erythroleukaemia development (Lakhanpal *et al.*, 2010), murine pancreatic cancer (Pilon-Thomas *et al.*, 2011) and myelodysplastic syndrome progenitor cells. Consistently, over-expression of SHIP-1 inhibits myeloid leukemic growth (Lee *et al.*, 2012). Therefore, similarly to PTEN, SHIP-1 has indeed been indicated as a tumour suppressive gene.

However, to suggest that SHIP-1 only functions to suppress PI3K dependent signalling is a vast simplification. The SHIP-1 product PI(3,4)P₂ has its own signalling properties such as the recruitment of DAPP1 (dual adaptor of phosphotyrosine and 3-phosphoinositides) and TAPPs (tandem PH domain containing protein) (Dowler *et al.*, 2000; Thomas *et al.*, 2001). Interestingly, low levels of PI(3,4)P₂ are potentially required for the full activation of Akt and Akt actually has a higher affinity for PI(3,4)P₂ than PI(3,4,5)P₃. SHIP-1 inhibition results in apoptosis of blood cancer cells which express SHIP-1 such as KG-1 (human myeloid cell line), suggesting PI(3,4)P₂ was actually required for their survival (Brooks *et al.*, 2010). In addition, PI(3,4)P₂ levels are increased in leukemic cells and increasing the level of PI(3,4)P₂ promotes the transformation and tumourigenicity of mouse embryonic fibroblasts. As such, there is currently significant interest in developing SHIP-1 inhibitors as a putative treatment of multiple myeloma; a disease with a high unmet medical need due to lack of effective treatment.

(b) Autoimmune or inflammatory disease

SHIP-1 is also proposed to be important in autoimmune and inflammatory diseases as evidenced by its role in: (1) immune cell development and homeostasis, (2) immune cell chemotaxis and (3) inflammatory mediator production.

The characterization of SHIP-1 knock out ($^{-/-}$) mice has provided a vital insight into its function in immune cells. SHIP-1 $^{-/-}$ mice are viable and fertile however their lifespan is significantly shortened with only 40% survival at 12 weeks. Interestingly, myeloproliferative disorder and massive infiltration of myeloid cells into the lungs is the main cause of the premature death (Helgason *et al.*, 1998). SHIP-1 deficient mice exhibit altered immune cell homeostasis with an imbalance between pro-inflammatory and anti-inflammatory myeloid and lymphoid cells. For example, SHIP-1 $^{-/-}$ mice have an increase in type 2 macrophages (M2) (Liu *et al.*, 1999).

In addition, comparing germline and conditional SHIP-1 null mice has been instrumental in identifying an important role of SHIP-1 in immune function (Helgason *et al.*, 2000; Wang *et al.*, 2002b; Tarasenko *et al.*, 2007; Leung *et al.*, 2009). In T lymphocytes, SHIP-1 has a crucial role in the development of Th17 cells and in regulating the balance between pro- and anti-inflammatory T lymphocyte populations (Locke *et al.*, 2009). Mice carrying a T lymphocyte-specific deletion of SHIP-1 uncovered a regulatory role for SHIP-1 in controlling Th1/Th2 bias and cytotoxic responses as a result of its inhibitory effect on T-bet expression. Hence, SHIP-1 null T lymphocytes do not skew efficiently to a Th2 phenotype and display Th1-dominant immune responses *in vitro* and *in vivo* (Tarasenko *et al.*, 2007). This is in contrast to evidence from germline SHIP-1 null mice, which indicates that SHIP-1 can also repress Th2 skewing by inhibiting IL-4 production from basophils. T lymphocyte-specific deletion of SHIP-1 using CD4CreSHIPflox/flox mice, had no effect on T-lymphocyte development, activation state or Treg-cell numbers (Tarasenko *et al.*, 2007). However, a recent study using in LckCreSHIPflox/flox mice reported significant reduction in the frequency of splenic CD3⁺ T lymphocytes and CD4⁺ and CD8⁺ T cells in the peripheral blood relative to SHIPflox/flox controls (Collazo *et al.*, 2012). The discrepancy may be because deletion of SHIP in CD4CreSHIPflox/flox mice may occur at a different time point during T-lymphocyte development compared to SHIP deletion in LckCreSHIPflox/flox mice.

PI3K-dependent Akt phosphorylation is vital in cells due to its importance in regulating inflammatory cell activation and survival. Reducing Akt dependent signals would be a method of reducing immune cell over-activation in autoimmune and inflammatory

disease. SHIP-1 overexpression reduces Akt phosphorylation and SHIP-1 deficiency markedly increases Akt phosphorylation in T lymphocytes, macrophages and mast cells. In addition, SHIP-1 activation decreases Akt phosphorylation in multiple immune cells types (Kerr, 2011). Consistently with their reduced Akt phosphorylation, over expression of SHIP-1 in macrophages, mast cells, T lymphocytes, B lymphocytes and dendritic cells elicit a reduced activation to bacterial mitogens. Likewise, SHIP-1 activation has been observed to reduce the secretion of inflammatory mediators GM-CSF, TNF- α , IFN γ and IL-2 in murine splenocytes as well as reducing LPS-mediated TNF- α release from macrophage (Leung *et al.*, 2009).

Immune cell migration is often observed to be dysregulated in immune pathologies. In addition, the ability of T cells to polarise and migrate is essential for the homing of lymphocytes to lymph nodes during immune-surveillance as well as to peripheral tissues during an immune response. PI(3,4,5)P₃ production is evident at the leading edge of polarized neutrophils (Wang *et al.*, 2002a; Ward, 2006), whilst PI3K is activated by most chemokine receptors expressed by T lymphocytes (Ward *et al.*, 2009) and is crucial in lymphocyte adhesion (Mazerolles *et al.*, 1996). Ectopic expression of constitutively active SHIP-1 into leukemic cells normally deficient in SHIP-1 impeded chemokine mediated signalling and migration (Wain *et al.*, 2005). Remarkably, the silencing of SHIP-1 expression in primary human T cells had no effect on directional migration, though basal migration was significantly impaired and this correlated with disassembly of microvilli and dephosphorylation of the actin associated proteins ezrin, radixin and moesin (Harris *et al.*, 2011).

Several opportunistic pathogens have exploited SHIP-1's regulatory role at inhibiting immune cell function to avoid immune detection. Gram negative bacteria *Actinobacillus actinomycetemcomitans*, which is involved in the pathology of meningitis and bronchopneumonia, avoids being detected by the immune system by producing Cytolethal distending toxin subunit (CdtB). CdtB mechanistically acts like SHIP-1, to dephosphorylate the 5' position of PI(3,4,5)P₃, which causes cell-cycle arrest and apoptosis (Shenker *et al.*, 2007). In a similar fashion, the measles virus produces SIP-110, a SHIP-1 homolog, to reduce PI(3,4,5)P₃ dependent T lymphocyte activation (Avota *et al.*, 2006). SHIP1 has been directly implicated in the pathogenesis of a variety of diseases including allergic inflammation, asthma, acute lung injury and inflammatory bowel disease (Kerr, 2011).

1.5.2 Pharmacological manipulators of SHIP-1

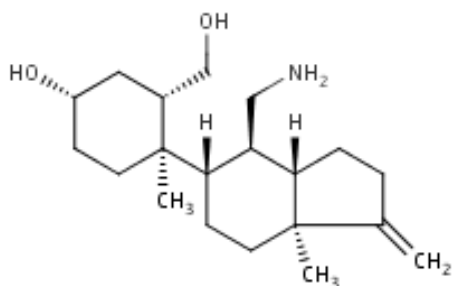
(a) SHIP-1 activators

To date, most genetic strategies to silence SHIP-1 protein expression have not been able to make a satisfactory distinction between effects caused by the ablation of SHIP-1 catalytic activity versus the removal of the various non-catalytic structural roles of SHIP-1 (Blunt *et al.*, 2012b). The SHIP-1 activators are known to target the C2 domain of SHIP-1, a region which binds the SHIP-1 catalytic product PI(3,4)P₂ inducing allosteric activation (Ong *et al.*, 2007). Adaptor functions of SHIP-1 are unlikely to be altered by pharmacological targeting and so, may offer the opportunity to discriminate between functions dependent on catalytic versus adaptor roles of SHIP-1.

The first SHIP-1 activator, named pelorol, was isolated from the marine invertebrate *Dactylospongia elegans* in 2005. The development of more potent and synthetic activators has since been undertaken by Aquinox Pharmaceuticals Inc, (Richmond, BC, Canada; (Ong *et al.*, 2007). Their lead compound AQX-1125 (1S,3S,4R)-4-[(3aS,4R,5S,7aS)-4-aminomethyl)-7a-methyl-1-methylidene-octahydro-1H-inden-5-yl]-3(hydroxymethyl-4-methylcyclohexan-1-ol; acetic acid salt [Figure 1.13]) reduces Akt phosphorylation in human leukemic T lymphocyte lines and murine B lymphocytes and importantly has been observed to inhibit several immune cells migration *in vitro*. Mechanistically, AQX-1125 was found to bind directly to SHIP-1, but failed to activate the enzyme when the C2 domain was deleted, suggesting its function is mediated through the C2 domain. In addition AQX-1125 was observed to activate SHIP-1 by inducing a concentration dependent reduction in the K_m of the enzyme, rather than affecting k_{cat} or V_{max}. Hence, Aquinox suggest that AQX-1125 induces a conformational change of SHIP-1, which results in better substrate binding and enzyme efficiency (Stenton *et al.*, 2013a). AQX-1125 has completed phase 2a trials for the treatment of mild to moderate asthma and significantly attenuated the late-phase response and improved FEV1 following allergen challenge (Meimetis *et al.*, 2012; Stenton *et al.*, 2013a; Leaker *et al.*, 2014). In addition, AQX-1125 is in two ongoing phase II trials for the treatment of interstitial cystitis /bladder pain syndrome and COPD (Table 1.1). Aquinox Pharmaceuticals are also recruiting participants for a Phase II pilot efficacy and safety study of AQX-1125 in atopic dermatitis [Aquinox Pharmaceuticals press release (Pharmaceuticals, 2015)].

(b) SHIP-1 inhibitors

The first SHIP-1 inhibitor was identified in 2010 using high throughput screening and termed 3 α -aminocholestane (3AC) (Brooks *et al.*, 2010) (Figure 1.13). 3AC increased the number of erythrocytes, neutrophils and platelets in myelosuppressed mice and increased the number of T regulatory cells which inhibited allogeneic T lymphocyte responses *in vivo*. Surprisingly, 3AC was observed to induce the apoptosis of leukemic cell lines and multiple myeloma cells. Given that the enzymatic product of SHIP-1, PI(3,4)P₂, has been shown to be able to activate Akt (Brooks *et al.*, 2010), this might be explained by the idea that a set amount of both PI(3,4,5)P₃ and PI(3,4)P₂ are required for optimal Akt activation. Therefore inhibiting SHIP-1 could disrupt the balance and promote cell apoptosis. At present the site at which 3AC binds to SHIP-1 is unknown.

A

AQX-1125

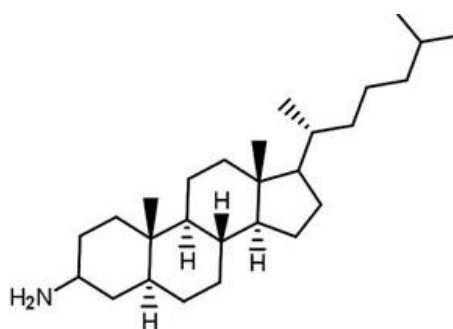
B3 α -aminocholestane (3AC)

Figure 1.13 Chemical structures of SHIP-1 modulators. (A) SHIP-1 activator AQX-1125 (Stenton *et al.*, 2013a) and (B) 3 α -aminocholestane (3AC) (Brooks *et al.*, 2010).

1.6 Reactive oxygen species in cell signalling

Reactive oxygen species (ROS) is the collective term for all reactive oxygen derivatives, and includes radicals such as superoxide ($O_2^{\cdot-}$) hydroxyl ($\cdot OH$) and peroxyl (RO_2^{\cdot}) and non-radicals such as hydrogen peroxide, hypochlorous acid (HOCl) and singlet 1O_2 . ROS are short-lived, highly electrophilic molecules generated by the partial reduction of oxygen. Some ROS are highly oxidising (e.g. OH, HOCl) and can form radical chain reactions; others are a rather mild oxidants (e.g. H_2O_2) (Winterbourn *et al.*, 2008). ROS species are known to induce bifurcate responses within cells. High concentrations evoke cell death and low concentrations are required for cell survival and signalling. Thus, regulation of ROS production must be very tightly controlled. The following sub-sections describe cellular sources and anti-oxidant defence mechanisms of ROS (Figure 1.14).

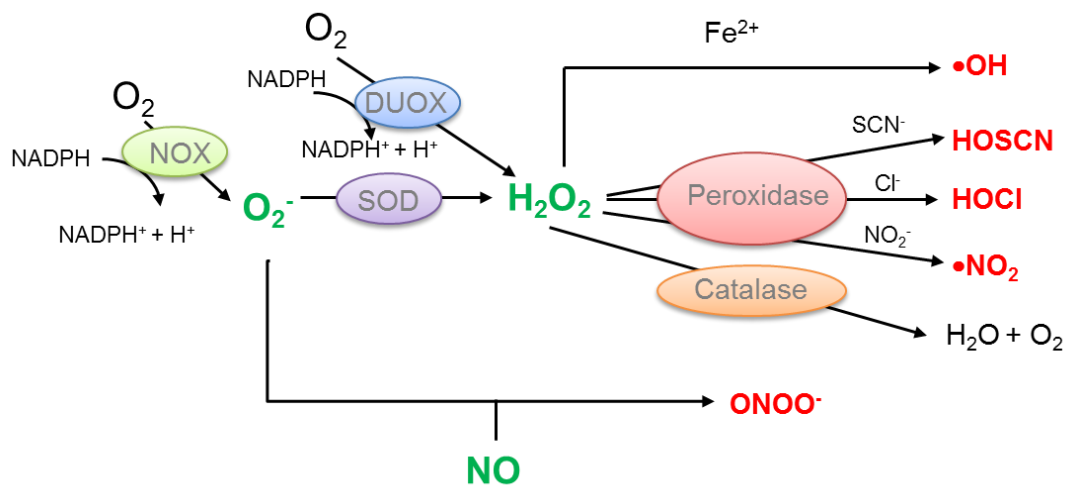


Figure 1.14 Reactive oxygen species. Superoxide ($O_2^{\cdot-}$) is generated from various sources, which include NADPH oxidase (NOX) and the mitochondria. Hydrogen peroxide (H_2O_2) is generated from dual oxidase (DUOX) enzymes and when two molecules of superoxide undergo dismutation, which is catalysed by superoxide dismutase (SOD). Catalase catalyses the breakdown of H_2O_2 into H_2O and O_2 . In the presence of iron (Fe^{2+}), superoxide and hydrogen peroxide react in a metal-catalysed reaction to generate hydroxyl radical ($\cdot OH$), termed the Fenton reaction. In addition, hypochlorous acid (HOCl), hypothiocyanous acid (HOSCN) and nitrogen dioxide ($\cdot NO_2$) are formed from hydrogen peroxide and peroxidase reactions. Nitric oxide (NO) can react with superoxide to form peroxynitrite ($ONOO^{\cdot-}$). The green colour indicates the ROS with reported signalling roles, whereas the red molecules are highly reactive and can induce cellular damage.

1.6.1 Sources of intracellular ROS

Various organelles within the cell can produce ROS, including the mitochondria, a by-product of aerobic metabolism, the endoplasmic reticulum from cytochrome P-450 and Ero1p enzymes (Gross *et al.*, 2006) and peroxisomes as part of their metabolism of long-chain fatty acids (Boveris *et al.*, 1972). In addition, there are many intracellular enzymes that generate ROS as part of their enzymatic reaction cycles, namely lipoxygenase (O'Donnell *et al.*, 1996), xanthine oxidase (McNally *et al.*, 2003), cyclooxygenase, cytochrome P450 monooxygenase, nitric oxide synthase and NADPH oxidase family (NOX). Mitochondria and NOX enzymes are described in more detail below.

(a) Mitochondria

The ROS generated in the mitochondria occurs during normal respiration and is generated at multiple sites in complex I and III. Early measurement of ROS production using isolated mitochondria *in vitro* suggested that ROS generation would account for approximately 1% of respired oxygen, indicating that mitochondria are the largest generators of ROS (Boveris *et al.*, 1972). However, recent estimates from mitochondria in living cells suggests normal mitochondria produce far lower levels of ROS (Nohl *et al.*, 2003). They likely provide a low basal level of ROS that can increase when the cell is highly active, ageing or exposed to hypoxia. In T lymphocytes mitochondria-derived ROS have been observed to be indispensable for the activation of NFκB, expression of cytokines and the CD95 ligand (Roth *et al.*, 2014). In addition, blocking mitochondrial ROS, specifically at Complex III result in the inhibition of CD3/CD28-induced IL-2 expression (Sena *et al.*, 2013)

(b) NADPH oxidase family (NOX)

NOX enzymes rapidly produce oxidants in response to receptor stimulation and thus have been termed professional ROS producers. Stimulation of several receptor including growth factors, Toll-like, cytokine and antigen receptors induce NOX enzyme-dependent ROS production, which is required for the activation signalling cascades (Ogier-Denis *et al.*, 2008). NOX enzymes are a family comprised of 5 NOX (NOX1-5) and 2 Dual oxidase enzymes (DUOX1-2) sub-types which differ in their expression, types of ROS released and mechanisms to control activity (Lassegue *et al.*, 2012). T

lymphocytes are known to express NOX2 (Jackson *et al.*, 2004) and DUOX1 (Kwon *et al.*, 2010), which are shown in Figure 1.15 with their corresponding regulatory subunits. NOX enzymes require interaction with regulatory subunits to allow their maturation and trafficking to the membrane, the 5 NOX subtypes require six different subunits that interact to form an active enzyme complex (Babior, 1999), whereas DUOXs associate with one other regulatory subunit. The activation of the NOXs is complex and requires small GTPase Rac, Rac1 (non-myeloid cells) and Rac2 in leukocytes, and phosphorylation events downstream PKC, Akt and PKA (Martyn *et al.*, 2005). Whereas, distinct to all other NOX enzymes, NOX5 and DUOX1/2 are directly activated by Ca^{2+} through EF hands (Sumimoto, 2008).

NOX enzymes are expressed both in plasma membranes and the membranes of intracellular vesicles, providing ROS production at localised sites. Indeed, receptors and signalling proteins have been observed to physically interact with NOX enzymes to form a complex, which could further enhance the specificity of signalling to the interacting receptor (Pawate *et al.*, 2004). NOXs are flavocytochromes (i.e. they contain both flavin adenine nucleotide and heme) and require electrons from cytosolic NADPH to reduce molecular oxygen, to generate superoxide which is rapidly dismutated to H_2O_2 by superoxide dismutase. DUOX enzymes directly generate H_2O_2 (Terada, 2006; Takac *et al.*, 2011).

In T lymphocytes, NOX has been shown to be an important signalling component in modulating differentiation into Th17 and Th1 lymphocytes. CD3 and CD28 ligation resulted in primarily Th17 phenotype in NOX-deficient mice, whereas the majority were the Th1 phenotype in NOX-intact cells (Tse *et al.*, 2010). In addition, T cells from mice deficient in the NOX catalytic subunit gp91 (phox) are more resistant to apoptosis (Purushothaman *et al.*, 2009).

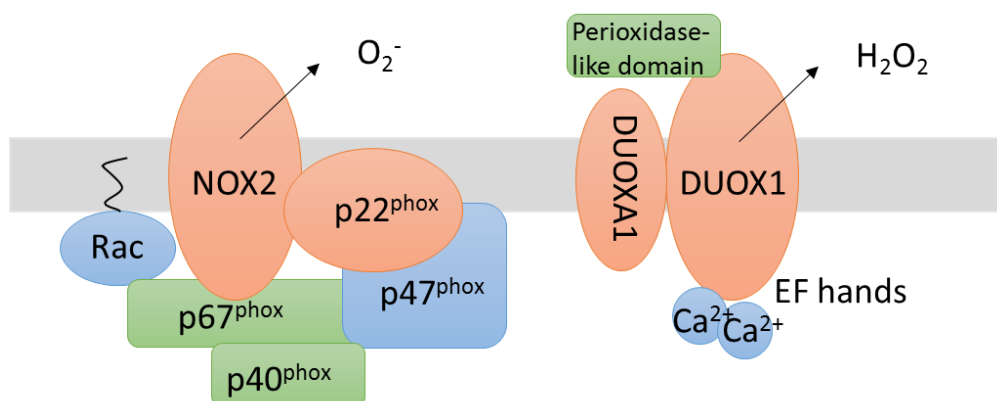


Figure 1.15 NOX2 and DUOX1 and their associated regulatory subunits.

1.6.2 ROS as a second messenger

To be able to function as a signalling agent ROS must have certain properties. Second messengers are required to be rapidly generated, and their concentration regulated by co-ordination of their production and destruction. There are several professional ROS producers and anti-oxidants within cells that regulate the rapid induction and accumulation and degradation of ROS (Section 6.1.1 & 1.3). Both superoxide and H₂O₂ have been indicated to signal, however it is generally accepted that H₂O₂ is the predominant signalling ROS. This is due to its greater stability and longer half-life of approximately 1ms allowing H₂O₂ time to encounter and react with signalling targets. H₂O₂ is also highly ubiquitous, small and can readily diffuse through aquaporin channels to access intracellular targets (Bienert *et al.*, 2006; Bienert *et al.*, 2007; Bienert *et al.*, 2014).

The traditional view was that ROS induces random and indiscriminate oxidation of intracellular lipids, proteins and DNA which led to cellular damage. However, more recently it was observed that H₂O₂ is able to induce reversible oxidation of specific protein targets. Sulphur containing amino acids, such as cysteine are the most easily oxidised (Janssen-Heininger *et al.*, 2008), however not all cysteine residues are equally susceptible which further enhances the selectivity of oxidative signalling. The *PKa* of each individual cysteine depends upon whether it is surrounded by acidic or basic amino acids and dictates whether the residue is more likely to exist as a thiol (SH) or more reactive thiolate anion (S⁻) at physiological pH. Thus, cysteine residues with low *PKa* (5.1-5.6) are more susceptible to oxidation.

Oxidation can alter charge, size and structural conformation of a protein which can in turn regulate a proteins signalling and indicates a role for ROS in divergent biological responses. The redox regulation of protein tyrosine phosphatases (PTPs) has been

widely studied and it is accepted that ROS inactivate PTPs. The consequence of PTP inactivation is the enhancement of un-regulated tyrosine phosphorylation by protein tyrosine kinases (PTKs) and the activation of downstream signalling (Denu *et al.*, 1998) (Figure 1.16). Interestingly, the selective oxidation of other signalling molecules has been observed and includes both activation and inhibition of kinases such as SFKs (Yoo *et al.*, 2011), MAPK (Galli *et al.*, 2008), Akt (Wani *et al.*, 2011), transcription factors (Haddad, 2002) and ion channels (Bogeski *et al.*, 2010). ROS are currently a rapidly expanding field of research due to high interest in determining mechanisms of oxidative signalling.

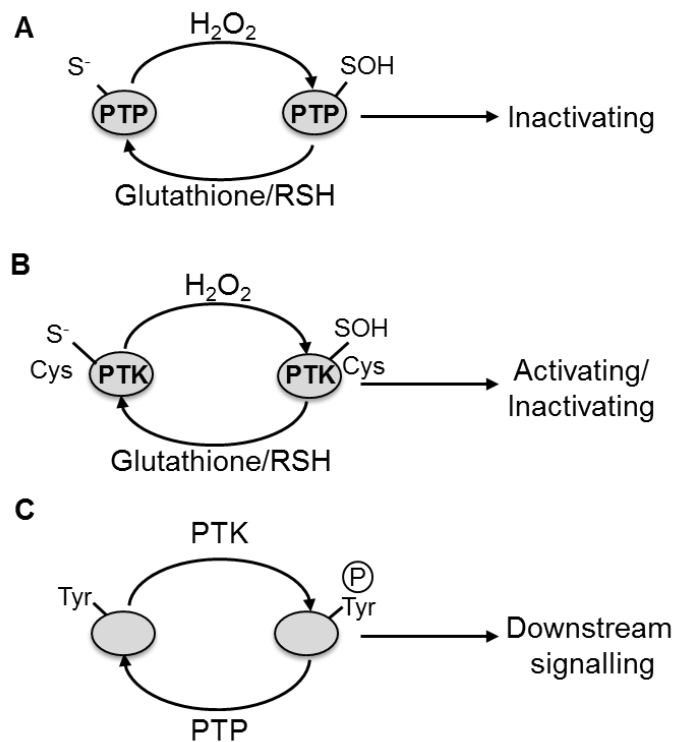


Figure 1.16 Redox dependent signalling. (A) Protein tyrosine phosphatases (PTPs) undergo oxidation of critical cysteine to inactivate their enzymatic activity, which can be reversed by glutathione or reducing thiols (RSH). (B) Protein tyrosine kinases (PTKs) can also undergo oxidation/regulation of critical cysteine residues which either activates or inhibits their activity. (C) The result of altering PTPs and PTKs activity changes the phosphorylation state of proteins and can lead to activation of their downstream signalling. Figure adapted from (Truong *et al.*, 2013).

1.6.3 Control of ROS accumulation within cells

To maintain ROS levels at an acceptable, non-damaging concentration cells have developed a variety of anti-oxidant mechanisms that commonly fall into two categories, enzymatic or non-enzymatic. The non-enzymatic are often small compounds often found in our food and drink, including ascorbic acid, vitamin E, glutathione, methionine, carotenoids and polyphenolic compounds (Seifried *et al.*, 2007). Cells can also increase expression of proteins and peptides which are cysteine rich to function as redox sinks when under oxidative stress, such as glutathione and peroxiredoxins. The enzymatic antioxidants include superoxide dismutase, catalase and peroxidases.

(a) SOD dismutase

Superoxide dismutase (SOD; (SOD E.C.1.15.1.1) is an anti-oxidant enzyme that catalyses the dismutation of highly reactive superoxide radicals into less reactive H_2O_2 and O_2 . SOD exists as three isoforms that have different cellular locations, cytosolic Cu/Zn SOD (SOD1), mitochondria Mn-SOD (SOD2) and SOD3 which is secreted to the extracellular environment. SOD enzymes contain copper, zinc, manganese or iron which facilitate electron transfer. SOD are biologically very important, SOD1 (-/-) mice exhibit a shortened life span and an increased incident of cancer. SOD1 mutations are observed in some patients with amyotrophic lateral sclerosis (Furukawa *et al.*, 2005). SOD2 (-/-) mice die soon after birth and develop a severe neurodegeneration disorder and SOD3 is important in preventing oxidative-stress induced diseases (Zou *et al.*, 2009). A T cell specific SOD2 conditional knock-out mouse exhibited increased superoxide production, apoptosis and development defects in the T lymphocyte population, resulting in immunodeficiency and susceptibility to influenza A virus H1N1 infection. This result indicates that the lack of mitochondrial SOD2 results in aberrant T cell development and function (Case *et al.*, 2011).

(b) Catalase

Catalase (EC 1.11.1.6) catalyses the decomposition of H_2O_2 to water and oxygen and thereby reduces the levels of H_2O_2 and prevents the formation of hydroxyl radicals via the Fenton reaction. The predominant catalase-containing organelle in mammalian cells is the peroxisome. A typical catalase is a tetrameric enzyme consisting of four heme containing identical subunits (Scibior *et al.*, 2006). Catalase is very important in protecting cells from oxidative stress. T lymphocytes transduced with catalase using a retroviral vector enhanced the survival of CD4⁺ T lymphocytes under oxidative stress (Ando *et al.*, 2008).

1.6.4 ROS in T lymphocytes

There is a growing body of evidence to suggest that reactive oxygen species (ROS) are actually required for a vast number of physiological processes, including TCR activation and signalling. In a concentration dependent manner, oxidation can elicit positive responses, such as cellular proliferation or activation, as well as negative responses, inhibiting growth inhibition and cell death (Figure 1.17).

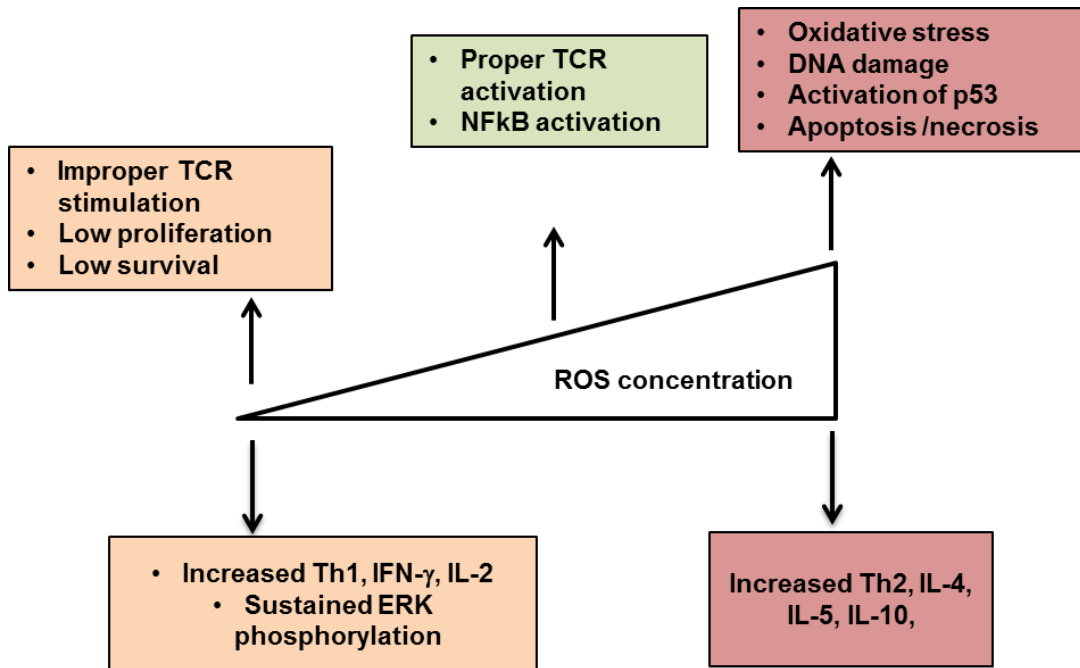


Figure 1.17 Fate of peripheral T lymphocytes to increasing concentrations of ROS. Low concentrations of ROS (colour coded orange) leads to improper TCR signalling with decreased proliferation and survival, whereas high concentrations (red) result in increased T lymphocyte death as a result of DNA damage. ROS concentration has been observed to affect the differentiation of T lymphocytes Adapted from (Kesarwani *et al.*, 2013)

ROS are critical for a wide spectrum of cellular processes including signalling, tumour progression and innate immunity. However, the roles of ROS in the human adaptive immune system development and function remain largely unknown. Recent studies have indicated that ROS affects the plasticity and differentiation of naïve T lymphocytes by altering IL-2 and IL-4 expression and cytokine secretion (Frossi *et al.*, 2008; Kaminski *et al.*, 2010; Tse *et al.*, 2010). In addition, the differentiation to different T lymphocyte subsets also appears to alter the cells relative sensitivity to oxidation. Interestingly, antioxidants inhibit T lymphocyte proliferation and IL-2 production in human and mice (Dornand *et al.*, 1989; Tatla *et al.*, 1999; Sena *et al.*, 2013). A very recent study has indicated that ROS is not required in TCR-induced proliferation,

activation and cytokine secretion of primary human T lymphocytes (Belikov *et al.*, 2014); before this study the roles of ROS and antioxidants on TCR signalling and responses had not been extensively investigated in primary human T lymphocytes isolated from the peripheral blood.

ROS production and scavenging has been detected using ROS sensitive fluorescent dyes. In T lymphocytes ROS is produced from a variety of stimuli, including mitogens (Williams *et al.*, 1996), viral and bacterial super-antigens (Weber *et al.*, 1995; Hildeman *et al.*, 1999), TCR peptide agonists (Devadas *et al.*, 2002; Kwon *et al.*, 2003) and CXCL12 (Hara-Chikuma *et al.*, 2012). ROS production has also been observed downstream of TGF β (Thannickal *et al.*, 2000), insulin (Mahadev *et al.*, 2001) and angiotensin II (Ushio-Fukai *et al.*, 1999). T lymphocytes are known to express NADPH oxidase enzymes NOX2 (Jackson *et al.*, 2004) and DUOX 1 (Kwon *et al.*, 2010) (Section 1.6.2). This implies that ROS might have a diverse and underappreciated role in the adaptive immune system.

1.6.5 H₂O₂ is a wound cue in *in vivo* models of inflammation

Generation of ROS, specifically H₂O₂, has been observed at sites of tissue damage across phylogenetically diverse organisms, including plants (Orozco-Cardenas *et al.*, 2001), *Drosophila* (Moreira *et al.*, 2010), zebrafish (Niethammer *et al.*, 2009) and mammals (Roy *et al.*, 2006). Historically it was thought that ROS was present at sites of tissue damage due to cell-rupture following injury or as part of an innate immune cell defence mechanism, termed respiratory burst, which provides toxicity to microbes attempting to enter the damage site. However, due to the recent identification of diverse biological signalling roles of ROS in immune cells, ROS has been intensely researched and observed to initiate leukocyte migration to wounds. Given the complexity of the human immune system and the inability to observe human immune cells in their intact environments (*i.e.* surrounded by maintained gradients of highly diffusible ROS species) scientists have relied upon animals to model inflammation and wound repair *in vivo*.

a) *In vivo* models of inflammation

Two important models of inflammation and wound repair have been utilised to indicate important roles of ROS in leukocyte recruitment. Briefly, zebrafish (*Danio rerio*) have many advantages for live imaging of leukocyte migration because their embryos develop externally and they are transparent allowing non-invasive observation of cellular behaviour *in vivo* by use of high-resolution imaging (Deng *et al.*, 2012). Furthermore, the immune system of zebrafish closely resembles that of mammals on a cellular and molecular level (Lieschke *et al.*, 2009). Tissue damage is achieved in zebrafish embryo by cutting the embryonic tailfin. Immune cell recruitment to tissue damage can be observed in transgenic zebrafish embryos with fluorescently labelled neutrophils and macrophages. A robust and rapid recruitment of neutrophils (Renshaw *et al.*, 2006), and a slower but more directional migration of macrophages to the tissue damage is observed (Ellett *et al.*, 2011). In addition, recent work has indicated that macrophages can repel neutrophils from inflamed tissues and are important in resolution of neutrophil mediated inflammation (Tauzin *et al.*, 2014) (Figure 1.18).

The second model uses fruit flies, *Drosophila melanogaster*, which also allow for live imaging of leukocyte migration as embryos are transparent and their genes are easily manipulated to study the genetics of inflammation and tissue repair. However *Drosophila* lack embryonic vasculature, mammalian chemokine orthologues and an adaptive immune system; instead they possess a very simplistic immune system which is easier to study. The main components the *Drosophila* immune system are hemocytes (macrophage-like innate immune cells). The wounding model in *Drosophila* consists of creating a laser induced epidermal wound (approximately 40µm diameter) in a late developmental stage 14 *Drosophila* embryo. Hemocytes within a further 20-30µm circumference abandon their previous position and are rapidly recruited to the wound site. The attraction of wounds is transient and within two hours post-wounding, hemocytes migrate back to their original locations. Similarly to the zebrafish model hemocytes can be fluorescently labelled and tracked by high resolution live imaging (Moreira *et al.*, 2010).

(b) *In vivo* models indicate a role for H₂O₂ as a wound cue

The mechanism of determining how H₂O₂ is produced at sites of tissue damage is not well understood. It was commonly believed that the main sources were ruptured cells after direct injury or inflammatory burst of innate immune cells, however new evidence using the *Drosophila* wounding model has indicated that ROS release requires a Ca²⁺ wave triggered in the epithelium. Reducing the Ca²⁺ signal with chelators impairs hemocyte migration to the wound and the Ca²⁺ signal was proposed to activate DUOX through EF hands in its extracellular domain to produce the H₂O₂ accumulation at the wound site (Razzell *et al.*, 2013) [Figure 1.18A].

In addition, a particularly eloquent study was performed using the zebrafish tailfin wounding model. Initially, zebrafish were genetically encoded with a H₂O₂ sensitive reporter, HyPer, to allow real-time spatial and temporal accumulation of H₂O₂ at a wound edge to be observed (Niethammer *et al.*, 2009). HyPer is highly selective for H₂O₂ over other ROS (Belousov *et al.*, 2006). A sustained rise in H₂O₂ was observed that started 3 minutes, peaked 17 minutes and was maintained until 61 minutes post wounding. The H₂O₂ formed a gradient about 100-200µm into the tailfin with a 50µM H₂O₂ concentration predicted at the wound edge. The presence of high H₂O₂ after 17 minutes indicated that the respiratory burst of the leukocytes could not be the source of the H₂O₂ as this represents the average time required for the first leukocyte which was not proximal to the wound edge to migrate into the vicinity of the wound. In addition, cell rupture is unlikely to maintain a H₂O₂ gradient over 60 minutes. Both the H₂O₂ gradient and leukocyte migration was lost in zebrafish embryos lacking DUOX or treated with NADPH oxidase inhibitor DPI, suggesting both H₂O₂ and DUOX are required for the recruitment of leukocytes to the wound site (Niethammer *et al.*, 2009) [Figure 1.18]. Consistently, a H₂O₂ gradient has since been observed to be required for hemocyte migration to a wound edge and treatment of the wound DPI inhibited leukocyte migration (Moreira *et al.*, 2010), consistently indicating that the H₂O₂ gradient is generated by DUOX.

(c) How does the cell detect the H₂O₂ gradient?

The mechanism by which H₂O₂ is detected by immune cells is not well established. Neithammer proposed the leukocytes could express H₂O₂ sensitive receptors. However alternatively H₂O₂ could manipulate innate cell migration through entering the cytoplasm and directly modulating intracellular mediators; several proteins have been indicated to be redox sensitive (Section 1.6.2). In particular, SFK member Lyn has been indicated as a redox sensor involved in early neutrophil recruitment to H₂O₂ at wounds. Mutation of a single conserved cysteine residue to alanine at position Cys466 abolished the ability of Lyn to be oxidised by H₂O₂. This mutation resulted in an inability of the cell to detect and migration towards the H₂O₂ gradient (Yoo *et al.*, 2011). Activation of Lyn leads to the activation of MAPK kinase and PI3K signalling pathways which are both required for neutrophil wound attraction. It has been speculated that asymmetric activation of PI3K signalling, by Lyn, could drive cell polarisation and migration along a H₂O₂ gradient (Yoo *et al.*, 2011) [Figure 1.18 B].

Cys466 is highly conserved across species (Zebrafish Lyn, *Drosophila* Src-42A and human SFK members) thus oxidation-dependent activation may be a general method of activation for all SFK members, however this has not been fully established. However ROS also activates the SFK, Fyn to aid the regeneration of wounded epithelium (Yoo *et al.*, 2012) and ROS activated-Yrk has been observed in macrophage migration to the wound site which is important in resolution of neutrophil driven inflammation (Tauzin *et al.*, 2014). Collectively these findings implicate ROS-SFK pathways as key regulators in multiple phases of the wound healing response. It is also not known how oxidation of SFKs alters their activity; however oxidation could provoke a conformational change in the protein.

In addition to inducing migration, DUOX induced H₂O₂ is required for the transcriptional activation of genes involved in epidermal tissue repair (Juarez *et al.*, 2011). In addition a mitochondrial ROS exhibits a cytoprotective role for in *Caenorhabditis elegans* skin epidermal repair. Local inhibition of mitochondrial ROS production blocked actin-based wound closure, whereas elevation enhances survival of mutant animals with defective wound healing. Xu indicates that mitochondrial ROS promotes wound closure by inhibition of redox sensitive Rho GTPase activity (Xu *et al.*, 2014). Interestingly, H₂O₂ has also been implicated in regulating leukocyte migration to transformed cancerous cells (Feng *et al.*, 2010).

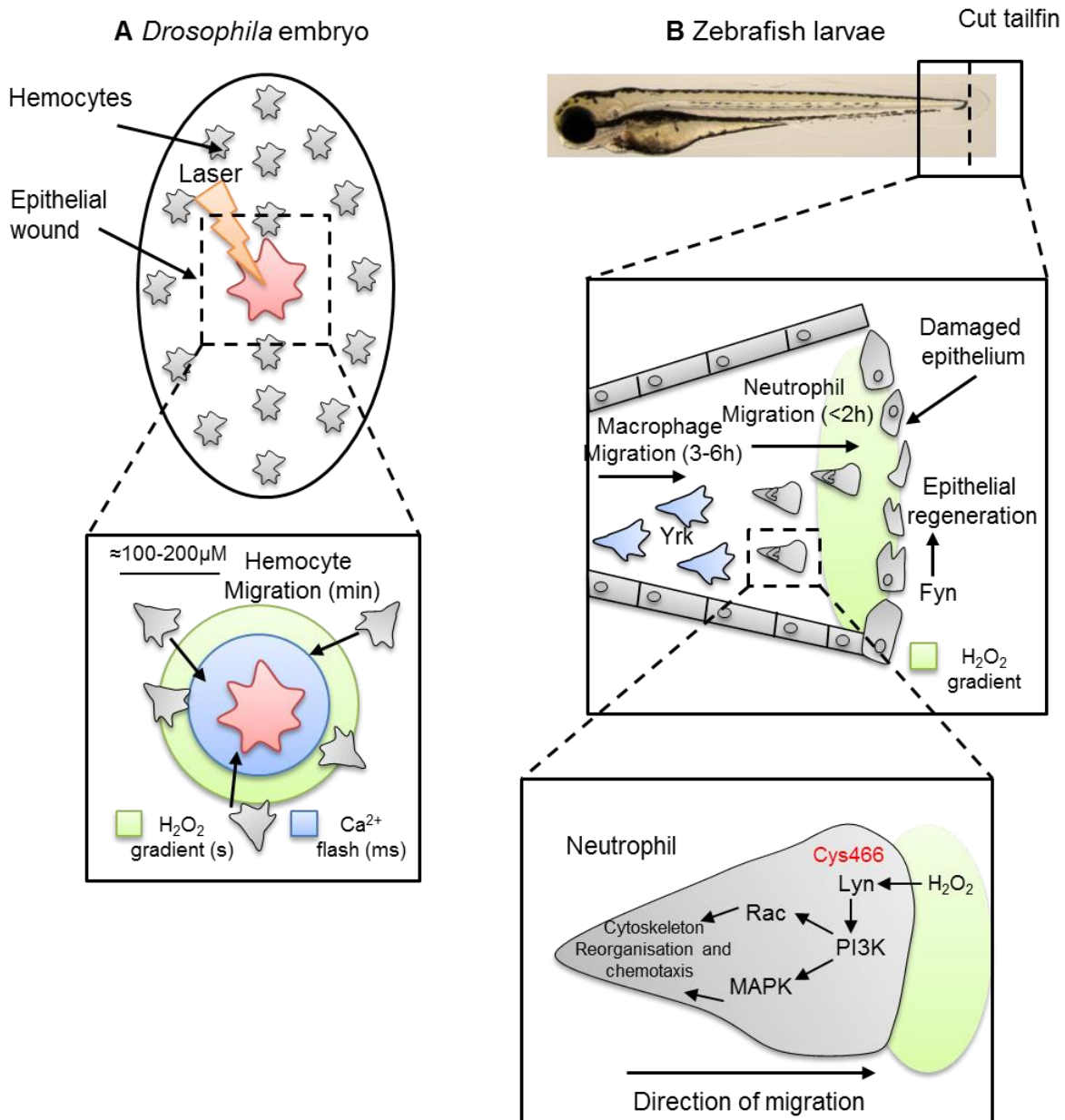


Figure 1.18 *In vivo* models indicate a role for H_2O_2 in leukocyte migration A) Laser ablation of *Drosophila* embryo epithelium drives the recruitment of hemocytes to the wound edge. Tissue damage triggers a rapid (ms) Ca^{2+} flash in epithelial cells proximal to the wound edge. This is followed by the production of a H_2O_2 gradient 100-200 μm from the wound edge which is maintained for at least 60 minutes post wounding. B) A cut of the zebrafish larva tailfin triggers the early recruitment of neutrophils (0-2h) followed by a more directional recruitment of macrophages (3-6h). Early neutrophil migration occurs along a gradient of H_2O_2 produced by DUOX enzymes in the damaged and surrounding epithelium. H_2O_2 oxidises Lyn kinase to activate signalling involved in cytoskeleton re-organisation and chemotaxis. Later macrophage arrival requires Yrk oxidation and is important in the resolution of neutrophil inflammation. Finally, ROS-activation of Fyn and genes transcription is important in epithelial regeneration (Adapted from Archer 2014).

1.6.6 H₂O₂ as a migratory cue for the adaptive immune system

H₂O₂ has been observed as a migratory cue in *in vivo* models of inflammation however, the organism used have a simplistic or immature immune systems and do not have functioning adaptive immune systems at the development stages investigated. In addition, they are clearly distinct from humans on an evolutionary scale. Interestingly, H₂O₂ has also been shown to act as a chemoattractant for mouse peritoneal neutrophils at low concentrations (Klyubin *et al.*, 1996). Furthermore, human neutrophils chemotaxis to gradients of H₂O₂ has been observed *in vitro* (Yoo *et al.*, 2011)

Thus H₂O₂ appears to be required for innate immune cell migration, however little is known about whether hydrogen peroxide is required for the migration of the adaptive immune system or human immune cells. Recently, H₂O₂ uptake, through aquaporin-3, was observed to be required for successful mouse T lymphocyte migration towards a chemoattractant, which suggests that H₂O₂ is also involved in the migration of adaptive immune cells. This H₂O₂ transport is essential for activation Cdc42 and subsequent actin rearrangement (Hara-Chikuma *et al.*, 2012). As H₂O₂ also appears to be important in the detection of transformed cells (Feng *et al.*, 2010) and ROS are involved in many other diseases (Section 1.6.8), it is particularly important to expand our knowledge on the effects of ROS in human T lymphocytes.

1.6.7 H₂O₂ and calcium mobilisation

Activation of DUOX has been recently linked to Ca²⁺ elevation and signalling. The intracellular loop of DUOX contains two canonical calcium EF hands, which bind cytosolic Ca²⁺ (Sumimoto, 2008). Section 1.6.5 describes that a Ca²⁺ wave precedes DUOX activation, H₂O₂ production and immune cell migration to a tissue damage site in the *Drosophila* embryo (Razzell *et al.*, 2013). In addition, DUOX has been shown to act in concert with SFKs and Ca²⁺ to signal and drive wound repair in zebrafish (Niethammer *et al.*, 2009; Yoo *et al.*, 2011).

Interestingly, exogenous H₂O₂ can induce calcium mobilisation and signalling, which has been postulated to be due to i) influx through voltage gated Ca²⁺ channels, ii) non-specific changes in membrane permeability, iii) alteration of Ca²⁺-Na²⁺ exchange iv) changes in Ca²⁺ release from intracellular stores (Krippeit-Drews *et al.*, 1995; Herson *et al.*, 1999; Wang *et al.*, 1999; Sato *et al.*, 2009). However, is important to establish the

concentration of H_2O_2 which causes necrosis in each cell type, as intracellular Ca^{2+} overload is an early marker of necrotic cell death (Zhivotovsky *et al.*, 2011)

1.6.8 ROS associated with disease

The link between oxidative stress and disease has been recognised for several decades. In disease conditions, natural human antioxidants defences are not always sufficient to maintain ROS balance which can lead to excessive and uncontrolled production of ROS. In particular, ROS are involved aging (Brieger *et al.*, 2012) as well as autoimmune diseases, leukaemia, carcinoma, cardiovascular diseases and neurological disorders (Kesarwani *et al.*, 2013). Interestingly, ROS and oxidative products are associated with the progression and severity of rheumatoid arthritis (Griffiths, 2005; Filippin *et al.*, 2008).

In light of recent experiments indicating that H_2O_2 acts to positively modulate immune cell migration (Section 1.6.5), ROS could be a factor in diseases presenting excessive immune cell recruitment (*i.e.* diseases that present with a high white blood cell count), such as asthma and COPD. Both these conditions are proposed to exhibit a high production of H_2O_2 due to chronically irritated tissues (Murata *et al.*, 2014). Cancerous cells often overproduce ROS, possibly due to elevated expression or overactive of NOX enzymes downstream of growth factor receptors. Oxidative stress is linked to genomic instability, chemo-resistance and growth mutation (Yang *et al.*, 2014). In addition, the link between inflammation and cancer has been suggested by epidemiological and experimental data and confirmed by anti-inflammatory therapies showing efficacy in cancer prevention and treatment (Bartsch *et al.*, 2006; Grivennikov *et al.*, 2010). Even the tradition Ayurvedic medical system, which was written 5000 years ago, described that continuous irritation over long periods of time could lead to cancer (Garodia *et al.*, 2007). Continuous irritation and wound healing are still a topic of intense interest in cancer research (Coussens *et al.*, 2002; Gonda *et al.*, 2009). Recent studies have focused on developing therapeutics to enhance the anti-oxidants within the cells and restore proper immune cell function. However clinical evidence and *in vivo* studies for not always support anti-oxidants reducing the inflammatory response (Higgins *et al.*, 1995; Gelderman *et al.*, 2007; Kesarwani *et al.*, 2013).

1.6.9 Hypoxia

Oxygen concentrations can become very low in pathological sites with low vasculature and sites of infection (Vaupel, 1977; Semenza, 2001; Via *et al.*, 2008). Under hypoxic conditions, mitochondria produce higher levels of ROS from the respiratory complex III (Guzy *et al.*, 2005). Therefore, in addition to being a relevant environmental setting for an activated T lymphocyte, exposure to hypoxia presents a method of manipulating the redox environment of the cell. Dysregulation of oxygen homeostasis is observed in inflammatory and cardiovascular disease, cancer, cerebrovascular disease and chronic obstructive pulmonary disease (Semenza, 2001).

Hypoxia inducible factor (HIF-1 α) is the key transcription factor mediating responses to hypoxia. In normal oxygen conditions, the alpha subunits of HIF are hydroxylated at conserved proline residues by HIF-prolyl-hydroxylases, allowing their ubiquitination, which labels them for rapid degradation by the proteasome. In hypoxic conditions HIF-prolyl-hydroxylases are inhibited, due to their use of oxygen as a co-substrate. HIF-1 α expression is stabilised and mediates the transcription of hypoxic sensitive genes to promote cell survival in low-oxygen conditions (Semenza, 2004).

1.7 Aims and Objectives

Adaptive immune responses are highly co-ordinated and rely upon efficient intracellular communication to induce cell functions. The experimental work in the thesis is divided into three sections which all focus on furthering our knowledge of signalling within human T lymphocytes. The first part dissects the roles of SHIP-1 in human T lymphocyte biology. The second focuses on the functional outcomes of ROS manipulation in human T lymphocytes and the final section examines signalling roles of H₂O₂ to explore the mechanisms underlying functional outcomes observed in part 2.

Both SHIP-1 and ROS-induced signalling and functional roles are not well understood in primary human T lymphocytes, with the majority of publications using animal cells, *in vivo* animal models or leukaemic cell lines. Both SHIP-1 activators and anti-oxidants are in clinical trials for the treatment of immune cell related pathologies, thus our intent is to improve the understanding of their signalling and define their potential as therapeutic targets for T lymphocyte driven pathologies such as autoimmune/inflammatory disease and cancer.

1. Determine whether SHIP-1 activator, AQX1, or SHIP-1 inhibitor, 3-AC, alter human T lymphocyte biology and indicate their therapeutic potential in T lymphocyte driven pathologies. Hypothesis:-SHIP-1 has pleiotropic functional roles in T lymphocyte function. The objectives to explore this hypothesis are to:

- establish the functional roles of SHIP-1 in human T lymphocyte viability and TCR activation including investigating downstream Ca^{2+} elevation, proliferation and adhesion.
- assess the functional importance of SHIP-1 as a regulator of basal and chemotactic migration, chemokine signalling, polarisation and cytoskeletal protein rearrangement.
- evaluate the potential of SHIP-1 as a novel therapeutic route to influence T lymphocyte driven inflammatory and autoimmune diseases.

2. Identify the functional roles of ROS manipulation in human T lymphocyte biology. Hypothesis: Non-toxic concentrations of H_2O_2 will activate survival, proliferation, signalling and migration in human T lymphocytes. The objectives to explore this area are to:

- establish the functional roles of ROS manipulation on ligand mediated cellular responses including viability, activation, downstream Ca^{2+} elevation, proliferation and adhesion using human T lymphocyte.
- determine whether human naïve or activated T lymphocytes migrate towards a H_2O_2 .
- establish the effect of ROS species upon directional T lymphocyte migration.

3. To examine the contribution of SFKs, SYKs, PI3K and SHIP-1 in ligand and H_2O_2 -mediated signalling in human T lymphocytes. Hypothesis: Activation of SFK is a key signalling event in H_2O_2 mediated activation of T lymphocytes. The objective to address this area are to:

- Analyse the contribution of SFKs, SYKs and PI3K isoforms in T lymphocyte chemotaxis using pharmacological manipulators.
- Interpret the responses of ROS manipulation on intracellular signalling and receptor expression.
- Dissect the signalling properties of SFKs, PI3K and SYK in response to H_2O_2 .

CHAPTER 2. Materials and Methods

2.1 Cell culture

2.1.1 Human leukemic T lymphoblast cell lines

Human leukemic T cell line Jurkat (American Type Culture Collection, ATCC; Manassas, Virginia, USA) were cultured in RPMI-1640 media (Invitrogen # 11875-093; Paisley, UK) supplemented with 10% (v:v) heat inactivated fetal bovine serum (FBS; Source Bioscience #7.01 Hi), 2mM L-glutamine [to provide energy supply for rapidly proliferating cells] 20mM HEPES (Sigma-Aldrich, Poole, UK) [a buffering agent used to maintain physiological pH] and 100U/ml of penicillin and 100µg/ml streptomycin (Invitrogen # 15140-122) [to protect against infection]. Culture flasks with a surface area of 75cm² were used to culture the cells at 37°C with a constant supply of humidified air containing 5% CO₂. The cell culture was maintained by changing the media every 2-3 days and cells were kept at a confluence of approximately 1 million cells per ml. Cells were discarded after 3 months of culturing and replaced with frozen cell stocks stored in cryo-tubes in liquid nitrogen tanks.

2.1.2 Peripheral blood mononuclear cells (PBMC) isolation from whole blood

Blood (50-100ml) was collected aseptically from healthy volunteers (donors were questioned to ensure they were not feeling unwell on the day of collection and volunteers with chronic health conditions were asked not to participate) of both sexes [who had not received any medication for at least 72h prior to donating] with a age range of 21-65, via a 21-gauge butterfly cannula (Bunzl Healthcare, Leicester, UK) into a sterile disposable 50ml syringe prepared with 2U/ml heparin (Sigma-Aldrich # 84020). The blood was subsequently diluted with warm sterile RPMI-1640 media without supplements in a ratio of 1:1. 25-35ml of the diluted blood was carefully layered onto 15ml of lymphoprep (Axis Shield # S1105, Dundee, UK) in 50ml plastic tubes (Figure 2.1.A). Preparations were spun at 250g for 30 minutes with braking deactivated upon deceleration for an unassisted stop (Figure 2.1.B). Following centrifugation, the mononuclear cell rich interface containing the PBMCs was removed by a sterile Pasteur pipette into a fresh 50ml falcon tube and diluted 1:1 with RPMI-1640 media. PBMCs were washed three times by centrifugation at 250g for 10 minutes, removal of the supernatant and addition of un-supplemented RPMI-1640 media to ensure complete removal of the lymphoprep solution.

2.1.3 Naïve CD4⁺ T lymphocyte isolation

“Naïve CD4⁺ T lymphocytes” were isolated from the freshly isolated PBMC using a naïve CD4⁺ T cell isolation kit II human (Miltenyi Biotec, Bisley Ltd., Surrey #130-094-131). This kit uses a negative selection method whereby a cocktail of biotinylated monoclonal antibodies (targeting CD45RO, CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD56, CD123, anti-TCR γ/δ , anti-HLA-DR, and CD235a /glycophorin A) indirectly label all the non-T-lymphocytes in the isolated PBMC (Figure 2.1). After washing, freshly isolated PBMC were counted using a haemocytometer. PBMCs were re-suspended in 40 μ l sterile ice cold MACS buffer (PBS + 2mM EDTA + 0.5% BSA, pH 7.4) per 10 million cells. 10 μ l of cocktail biotinylated monoclonal antibody per 10 million cells was then added for 10 minutes at 4°C to label all non-naïve CD4⁺ cells. 10ml MACS buffer was added and cells centrifuged at 250g for 10 minutes before supernatant was completely removed and cells re-suspended in 80 μ l ice cold MACs buffer and 20 μ l biotinylated micro-beads per 10 million cells for 15 minutes (Figure 2.1). After which another 10ml of ice cold MACs buffer was then added and the cells centrifuged at 250g for 10 minutes. The supernatant was discarded and cells re-suspended in 500 μ l fresh MACs buffer. An LS column [#130-042-401] was prepared by washing through 3ml of ice cold MACs buffer. The cells were then added to the column and output collected in a 15ml falcon tube. The column was then washed three times by the repeated addition of 3ml MACs buffer (Figure 2.1). Cells were centrifuged and re-suspended into supplemented RPMI-1640 media for immediate use.

2.1.4 Clonal expansion of CD4 and CD8 T-lymphocytes

To clonally expand CD4⁺ and CD8⁺ lymphocytes, the washed PBMCs were re-suspended in fully supplemented RPMI-1640 media at an equal volume to that of the original volume of whole blood collected from the donor. *Staphylococcal enterotoxin B* (SEB; Sigma # S4881) was added at a final concentration of 1 μ g/ml for 72 hours. The suspension cells were then collected in a 50ml falcon and washed three times in un-supplemented RPMI-1640 media and placed in a fresh cell culture flask the same volume of complete media supplemented with interleukin-2 (IL-2; Chemicon; Hampshire, UK) at 36U/ml. Any contaminating adhering cells (such as monocytes) were removed by plastic adherence to the original flask. The cells were washed, re-suspended and supplemented with fresh IL-2 every two days for 10-14 days maintain cell confluency between 0.1 to 1 million cells per ml. Cells were used 8-14 days post isolation and rested in complete media without IL-2 overnight before use.

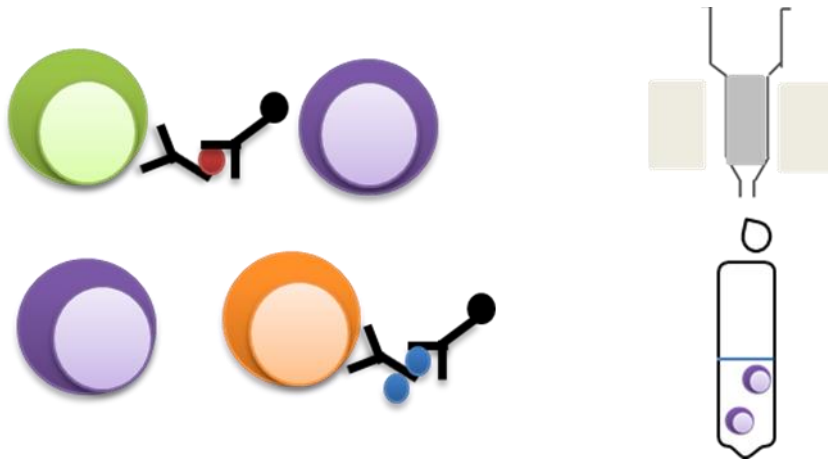


Figure 2.1 Isolation of naïve T lymphocytes from whole blood. Left hand panel is a schematic representing non-naïve CD4⁺ T lymphocytes (in green and orange) bound by a cocktail of biotinylated monoclonal antibodies (targeting CD14, CD16, CD19, CD36, CD56, CD124) and anti-biotin (avidin) coated micro-beads. Naïve CD4⁺ T lymphocytes are untouched and represented in purple. Right hand panel is a schematic representing the naïve T lymphocytes passing through a LS column selection, whilst cells bound to the beads will remain inside the magnetic column (Original graphic).

2.2 Cellular viability assays

2.2.1 Propidium iodide and annexin V staining

SEB activated T lymphocytes were washed three times into un-supplemented media. Cells were treated with compounds as stated in the figure legends for 3 hours. Cells were then re-suspended at 1 million cells per ml in Annexin V binding buffer (10mM HEPES, 140mM NaCl 2.5mM, CaCl₂, 0.1 % (w:v) BSA; pH 7.4). Cy5 conjugated Annexin V (Life Technologies) was then added at 10µl per 1 million cells for 20 minutes on ice protected for light. After 20 minutes, 2.5µg/ml propidium iodide PI (Sigma Aldrich # 81845) was added to the cells and fluorescence emission read immediately using flow cytometry and emission read at 488nm (Cy5) and 617nm (PI).

2.2.2 MTT assay

Cells were re-suspended in fresh complete RPMI media at 1 million cells per ml. 100µl of cells were added in triplicate for each condition to a 96 well-plate. Triplicate wells were treated for either 3 or 24 hours with either vehicle or stated concentrations of compounds at 37°C. The plate was centrifuged at 250g for 10 minutes and supernatant aspirated. Cells were re-suspended in 100µl MTT mix (RPMI, 10% FBS and 500µg/ml 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) Sigma Aldrich # M2003) and incubated for 3 hours. Background absorbance was determined from a well containing only the MTT mix. In living cells, yellow MTT is reduced by mitochondrial succinic dehydrogenases to blue formazan; fluorescence intensity is therefore proportional to the number of living cells. After 3 hours 100µl DMSO (Sigma Aldrich # D8418) was added to each well to lyse the cells. Absorbance at 540nm was then read using a FluoStar Optima plate reader

2.3 Proliferation

Cell proliferation was measured using the Cell Trace CFSE Cell Proliferation Kit (Invitrogen #C34554) according to the manufacturer's instructions. Carboxyfluorescein diacetate N-hydroxysuccinimidyl ester (CFSE) is a cell permeable, non-fluorescent pro-dye. Intracellular esterases cleave the acetate groups which results in fluorescent carboxyfluorescein which is membrane impermeant. The succinimidyl ester groups binds covalently to intracellular amines and emit fluorescence at 517nm when excited at 492nm. The result is live fluorescently labelled cells. As the cells divide the CFSE is partitioned between the two daughter cells. Hence, by tracking mean CFSE fluorescence intensity over time cell proliferation can be determined.

Freshly isolated naïve CD4⁺ T lymphocytes were re-suspended at 1 million cells per ml in PBS and 0.1% BSA. CFSE was added for a final concentration of 5µM and cells incubated at room temperature in the dark for 15 minutes. Cells were washed once into fresh RPMI and incubated at 37°C for 30 minutes. Cells were washed again into fresh complete RPMI media at 1 million cells per ml. Freshly labelled naïve T lymphocytes were taken and analysed by flow cytometry to ensure dye incorporation and determine mean fluorescence intensity on Day 0. The remaining cells were treated with vehicle or stated concentrations of drugs. Cells were then activated by the addition of anti-CD3/CD28 coated beads (methods described below) at a ratio of 3 beads to each cell and 36U/ml recombinant IL-2. After either 4 or 5 days (stated in the figure legends) cells were analysed for CFSE fluorescence using flow cytometry, excitation at 492nm and emission at 517nm. Population gates were used to determine the percentage of cells in each cell division observed.

2.3.1 Coupling of anti-CD3/CD28 antibodies to micro-beads

75µg of antibodies raised against human CD3 (clone UCHT-1) and human CD28 (clone 9.3) were added to a 0.1M borate buffer (pH 9.5). This antibody cocktail was added to 400 million magnetic Dynabeads (Invitrogen) and rotated overnight at 37°C. Then the beads were held with a magnet and the borate buffer removed. Using the bead wash solution (Phosphate buffered saline without Ca²⁺ and Mg²⁺, 3% (v:v) human AB serum, 1% (v:v) sodium azide, 2mM EDTA pH 8.0) and beads were washed three times for 10 minutes and once for 30 minutes and then overnight at 4°C whilst rotated. The following day beads were re-suspended in 10 ml bead wash solution. Storage of Anti-CD3/CD8 coated beads at 40 million per ml at 4°C. Before use, beads were washed three times in phosphate buffered saline, re-suspended in RPMI-1640 and added to the cells at indicated ratios.

2.4 Cell stimulation protocol

Cells used for stimulations were re-suspended at a concentration of 2 million cells per ml in supplement free RPMI-1640 media with HEPES (1mM) and rested for 30 minutes in a 37°C water bath. Vehicle control (Ethanol/DMSO as appropriate at the same concentration as present in the highest drug treatment), drug treatments were carried out for the indicated times before the additions of stimulants. After stimulation, cells were placed immediately on ice, washed once in ice cold PBS before cell lysis for Immunoblotting.

2.4.1 Cell lysis

Stimulated and PBS washed cells were then centrifuged for 1 minute at 250g, the supernatant was discarded and cell pellet re-suspended in 50µl lysis buffer (50mM Tris-HCl [pH 7.5], 150mM NaCl, 1% (v:v) Nonidet-P40 [BDH], 5mM EDTA, phosphatase inhibitors, 1mM sodium vanadate, 1mM sodium molybdate, 10mM sodium fluoride and protease inhibitors 40µg/ml PMSF, 0.7µg/ml pepstatin A, 10µg/ml aprotinin, 10µg/ml leupeptin, 10µg/ml soybean trypsin inhibitor [All from Sigma]). Samples were rotated for 30 minutes at 4°C to assist cell lysis after which cells were centrifuged 153g, 4°C for 10 minutes. The supernatant containing protein was removed and insoluble material discarded. 5µl 5× sample buffer (60mM TRIS-HCl, 2% [w:v]SDS, 10 % [v:v] glycerol, 5% [v:v] 2-mercaptoethanol and 0.01% bromophenol blue sodium salt; pH 6.8) was added to 20µl protein in lysis buffer and boiled at 100°C for 5 minutes. Samples were briefly centrifuged at 250g. Supernatant containing protein and boiled samples containing sample buffer were stored briefly at -20°C before Immunoblotting.

2.4.2 Immunoblotting

Proteins were separated using 10% (w:v) acrylamide gels

Component	Stacking gel (5%)	Resolving gel (10%)
0.5 M Tris/HCl + 1% SDS, pH 6.8	0.75ml	
1.5 M Tris/HCl + 1% SDS, pH 8.8	-	1.25 ml
1% (w:v)Ammonium persulphate (AMPS)	37.5 μ l	37.5 μ l
30% (w:v) Acrylamide	0.5 ml	1.67 ml
milliQ H ₂ O	1.708 ml	2.04 ml
Temed (Sigma)	3.75 μ l	3.75 μ l
Total volume	3 ml	5 ml

Table 2.1 Composition of a 10% (w:v) SDS-acrylamide gel.

10% SDS acrylamide gels were prepared using the Mini PROTEAN Tetra Electrophoresis System (BioRad, Hemel Hempstead, UK). The resolving gel (Table 1) was cast first and milliQ water gently layered on top. The gel was left to polymerize for approximately 20 minutes. Once the resolving gel has set the water was poured off and replaced with stacking gel (Table 2.1) containing a 10 or 15 well comb to create the wells for protein loading. The stacking gel was left to set for another 20 minutes. Once set the comb was removed and wells cleaned with milliQ water to remove non-polymerized acrylamide. The gel was then loaded into the apparatus and tank filled with running buffer (250mM Tris base, 1.92M Glycine and 1% (w:v) SDS). 20 μ l of sample and 2 μ l EZ-run protein ladder (Gibco) were then loaded and electrode lid attached to the tank. The gels were run at 75V for 15 minutes or until the proteins had passed through the stacking gel. The voltage was then increased to 150V to separate the proteins by electrophoresis through the resolving gel until the dye in the loading buffer emerged from the bottom of the gel.

2.4.3 Semi dry protein transfer

Protein was transferred to a nitrocellulose membrane using a semi dry transfer protocol. Firstly the graphite electrodes of the transfer apparatus were dampened with semi-dry transfer buffer (480mM Tris base, 390mM Glycine, 0.375% (w:v) SDS and 20% (v:v) methanol). Whatman paper (Fisher) and the nitrocellulose membrane (GE Healthcare UK) were cut to the same size as the gel and pre-soaked in semi-dry transfer buffer. 3 pieces of Whatman paper were layered on the graphite electrode, and the nitrocellulose membrane was placed on top and gently rolled to remove air bubbles. The gel was carefully placed on top and covered by another 3 pieces of Whatman paper which again was rolled to expel air bubbles. The transfer was run for 1 hour at 0.8A/cm². The nitrocellulose membrane was blocked from non-specific antibody binding by incubation in either 2% (w:v) BSA in TBS-Tween (200mM Tris Base, 1.5M NaCl and 0.05% (v:v) Tween pH 7.4; Sigma-Aldrich) or 5% (w:v) milk in TBS-Tween. The membrane was then washed briefly in TBS-Tween to remove excess blocking buffer and incubated at 4°C overnight with the relevant primary antibody (Table 2.2).

Primary antibody	Host species	Concentration (µg/ml)/ dilution factor	Source
p-Akt ^(Serine 473)	Rabbit	1:1000	Cell Signalling Technology #4060
ERK1/2	Rabbit	0.2	Santa Cruz Biotechnology #sc-93
p-ERK1/2	Rabbit	1:1000	Cell Signalling Technology #9101
p-ERM	Rabbit	1:1000	Cell Signalling Technology #3141
p-Src	Rabbit	1:1000	Cell Signalling Technology #2101
Src	Rabbit	0.2	Santa Cruz Biotechnology #sc-18
p-SHIP-1	Rabbit	1:1000	Cell Signalling Technology #3941
SHIP-1	Rabbit	1:1000	Cell Signalling Technology #2725

Table 2.2 Primary antibodies used for Immunoblotting. For Cell Signalling Technology antibodies dilution factors are reported as the initial antibody concentration is not available. Primary antibodies are diluted in 5ml TBS-Tween, 0.01% (w:v) BSA and 0.001% Sodium Azide.

The membranes were washed 3 times for 5 minutes in TBS-Tween to remove any excess primary antibody and then exposed to the secondary antibody 0.001% (v:v) HRP conjugated anti-rabbit IgG (Dako #P0448). The membranes were incubated for 1-2h with the secondary antibody and washed again 3 times for 5 minutes in TBS-Tween. Membranes were then placed in Chemiluminescent Lumigen reagent (Amersham Bioscience) for 1 minutes and then exposed by X-ray film (Fuji Medical X-Ray Film 100 NIF 18x24; Fisher Scientific) inside a film cassette for between 30sec-2hr and then developed using a film developer (Photon Imaging Systems, Swindon, Wilts, UK). Band intensity was quantified using ImageJ, whereby the rectangular selection tool from the Image J toolbar was used to detect set areas around each band. The pixel intensity of each rectangle was measured and calibrated against the pixel intensity of the background.

Alternatively, nitrocellulose membranes were placed in Chemiluminescent Lumigen reagent (Amersham Bioscience) for 1 minutes and then imaged using an ImageQuant developer (GE Healthcare, Life Sciences). Band intensity measured using 1D gel ImageQuantTL analysis software version 7.

Alternatively, nitrocellulose membranes were blocked for 2h in 5% (w:v) milk then placed in fluorescence secondary antibody (LICOR Donkey anti Rabbit) (1:5000) in TBS tween 1% (w:v) BSA. Blots were then washed 4 times for 5 minutes in TBS tween then once in PBS before fluorescence intensity at 680nm was imaged using the Licor Infrared developer (Odyssey).

2.4.4 Membrane stripping and reprobing

The membranes were placed in a sealed container including at least 25ml of stripping buffer (100mM 2-Mercaptoethanol, 2% [w:v] SDS, 62.5mM TRIS-HCl; pH 6.7) and placed in a water bath at 60°C for 30 minutes. The membrane was then washed 6 times over the course of 1hr in TBS tween. The membranes were then blocked for 1hr in 5% (w:v) milk in TBS tween and then exposed to a different primary antibody.

2.5 Immunoprecipitation of SHIP-1

By immunoprecipitating SHIP-1 from human T lymphocytes the compounds can be assessed directly on the catalytic activity of SHIP-1. SHIP-1 was immunoprecipitated from the SEB activated T lymphocytes. Cells were centrifuged 250g and re-suspended in serum free RPMI-1640 media. 1 million cells were lysed for used for each reaction of the malachite green assay. Samples were rotated for 30 minutes at 4°C to assist cell lysis after which cells were centrifuged 153g, 4°C for 10 minutes. The supernatant containing protein was removed and insoluble material discarded. After centrifugation the supernatant was incubated overnight in 0.01% (v:v) SHIP-1 monoclonal antibody (Cell Signaling Technology). A/G Agarose beads (Peprotech) (5µl per point of malachite green) were washed twice in lysis buffer without inhibitors, re-suspended in 100µl and added to the cell lysis of the MOLT-4 cells for at least 2 hours. Samples were then washed 3 x in 1ml lysis buffer and 3 x in malachite green reaction buffer (5% (v:v) glycerol, 20mM Tris HCl, 10mM MgCl₂; pH 7.4) to remove all proteins not attached to the A/G agarose beads and re-suspended in 20µl of reaction buffer/point.

2.5.1 Malachite green assay

The immunoprecipitated SHIP-1 was then used in the malachite green phosphate assay. Full length recombinant SHIP-1 (Gift from GlaxoSmithKline) was diluted to 1µM in reaction buffer and assessed in the malachite green reaction. SHIP-1 can dephosphorylate the *in vitro* substrate D-Ins(1,3,4,5) tetrakisphosphate to produce Ins(1,3,4)P₃ and free phosphate. A malachite green solution can react with the free phosphate to give a rapid colour change from yellow to green. 20µl of immunoprecipitated SHIP-1 solution, or 1µM recombinant SHIP-1 was added to each of the required wells of a 96 well plate and incubated with SHIP-1 activators, inhibitors or H₂O₂ at given concentrations for 5 minutes at room temperature. 100µM substrate D-Ins (1,3,4,5)P₄ (Echelon Biosciences, Salt Lake City, USA #Q-1345) was added for 30 minutes at 37°C. 100µl malachite green solution (Echelon, # K-1501) was added to each well and incubated for 15 minutes at room temperature in the dark. The plate was then read at 650nm using a FluoStar Optima plate reader.

2.6 Flow cytometry

Freshly isolated naïve CD4⁺ and SEB-activated T lymphocytes were washed three times by centrifugation at 250g and re-suspended in non-supplemented RPMI-1640 media. After treatment with vehicle or activator as described in the figure legends, cells were fixed using BD Fixation and Permeabilization solution (containing formaldehyde; BD Biosciences, Oxford, UK) for 30 minutes at 4°C to allow intracellular staining of proteins. Cells were washed twice in BD PERM/Wash solution (containing sodium azide and saponin; BD Biosciences) solution and incubated with 0.02% (v:v) anti-phospho ERM (Cell Signalling Technology #3141), anti-phospho-SHIP-1 (Cell signalling #3941) diluted in BD PERM/Wash for 30 minutes at 4°C. Cells were washed twice by centrifugation at 250g and re-suspension BD PERM/Wash solution and then incubated with 0.01% (v:v) anti-rabbit IgG FITC conjugated secondary antibody (Sigma #F9887) for 30 minutes at 4°C. Alternatively, cells were stained with TRITC conjugated phalloidin for 30 minutes. Cells were washed twice in BD PERM/Wash solution, re-suspended in 400µl ice cold PBS + 1% (v:v) FBS in 5ml rounded bottom FACs tubes (BD Falcon, Willford, Nottingham) for analysis by BD FACS Canto II flow cytometer and BD FACS DIVA software (BD Biosciences). TRITC stained cells were excited at 547nm and emission recorded at 573nm whereas FITC stained cells were excited at 495nm and emission recorded at 519nm. Mean fluorescence index (MFI) was used to describe the level of phosphorylated ERM, SHIP-1 protein or actin polymerisation.

2.6.1 Surface receptor expression by flow cytometry

SEB activated T lymphocytes were re-suspended in fully supplemented RMPI-1640 at a concentration of 1 million cells per ml. Cells were treated with either vehicle or stated concentrations of drugs for 30 minutes. Cells were washed twice with ice cold FACs buffer (PBS + 2% FCS) then re-suspended in diluted 1/50 (v:v) phycoerythrin (PE) conjugated anti-CXCR3 antibody (R&D biosciences # FAB16OP) or PE conjugated IgG isotype control (1/50 v:v dilution; R&D biosciences #1COO2P). Alternatively cells were labelled with either Anti-human CD11a PE-conjugated monoclonal antibody (1/50 v:v dilution; ImmunoTools # 21270114), anti-human CD49d PE-conjugated monoclonal antibody (ImmunoTools # 21488494) or PE conjugated IgG antibody. Cell were labelled on ice for 1 hour then washed twice more in FACs buffer. Alternatively, cells were incubated with primary antibody 10µg/ml KIM127 mAb (Gift from Nancy Hogg) for 30 minutes then washed and incubated to secondary anti-mouse FITC conjugated antibody (dilution 1/50 v:v). Finally, cells were re-suspended in PBS and placed in 5ml

round bottomed tubes (VWR, Lutterworth, Leicestershire, UK) for analysis by flow cytometry. PE-fluorescence was detected by exciting cells at 496 nm and emission recorded at 576 nm recorded by BD FAC Aria flow cytometer and analysed by BD FACs Diva Software. Mean fluorescence index (MFI) was used to describe the level of CXCR3, CD11a and CD49d expression.

2.7 *In vitro* cell migration assays

2.7.1 Neuroprobe assay

A Boyden chamber 96 well plate assay can be used to determine *in vitro* cell migration. Cells were washed three times in non-supplemented RPMI-1640 medium, re-suspended at 3.2 million cells per ml in phenol red free RPMI-1640 media and treated with either vehicle or inhibitor at the indicated concentrations for 30 minutes. Whilst preparing the cells, a 96 well Neuroprobe chemotaxis plate (Chemo® Tx101-5; Receptor Technologies, Warwick UK; [Figure 2.2 A]) was equilibrated by the addition of 29µl of PBS to the lower chamber and incubation at 37°C for 1 hour. PBS was aspirated from the wells and replaced with either chemotaxis media (phenol red free RPMI-1640 media; Invitrogen) or chemokines CXCL11, CXCL12 or CXCL10 (Peprtech, London UK) diluted in chemotaxis media. This was overlaid with a supplied 5-µm pore-size filter membrane and 25µl of the cell suspension (total cell number 80,000) was added over each required well (Figure 2.2 B). The plate was then incubated at 37°C in 5% CO₂ for 3 hours. A single piece of Whatman blotting paper was then passed evenly over the membrane to remove the cells which have not moved from the top 25µl cell suspension. The plate was then centrifuged at 250g for 10 minutes with the brake deactivated to allow an unassisted stop. This allows the cells which have migrated through the membrane to be collected in the bottom well. The membrane was carefully removed and cells re-suspended from each well and placed into 300µl PBS. Cells were then counted for 30 seconds for each well by flow cytometry whereby cells were selected by a gate to eliminate debris.

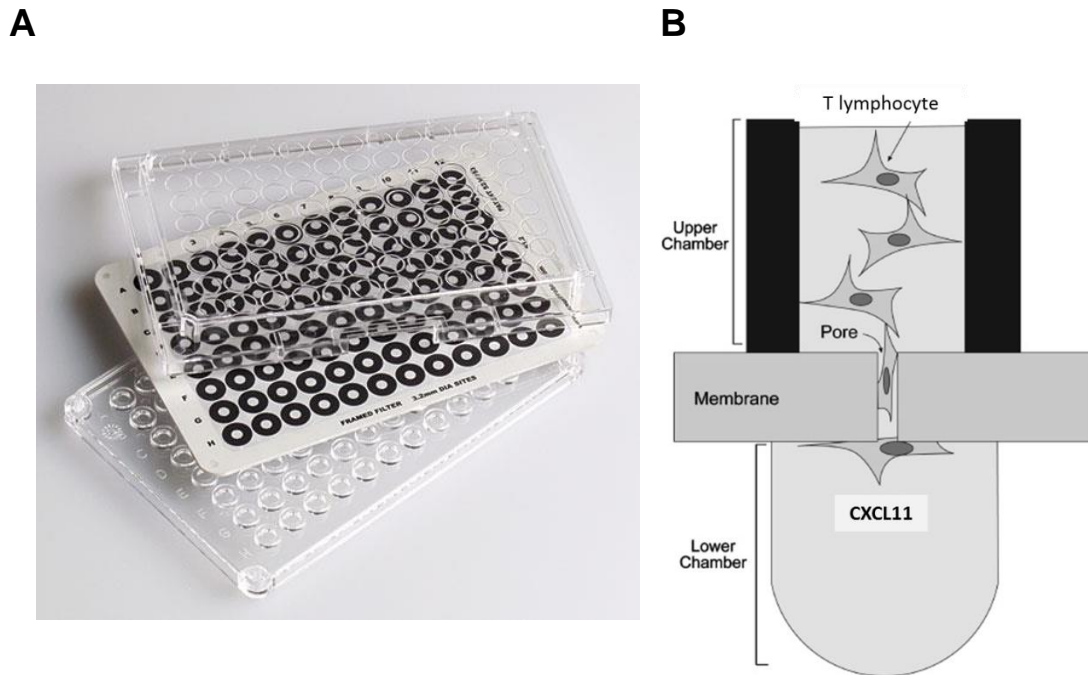


Figure 2.2 Apparatus and schematic for determining *in vitro* cell migration. A. Photograph of the apparatus supplied by Receptor Technologies to undertake cell migration. The 96 well plate system contains a lower chamber containing plate, a sheet containing 96 circular membranes with 5µm pores surrounded by black hydrophobic rings and a plastic lid. B. Schematic side on view of the preparation of a Neuroprobe experiment. The cell suspension is placed in the upper chamber, if cells are able to migration they move towards the chemokine CXCL11 in the lower chamber. Cells are retained in the lower chamber and counted by flow cytometry.

2.7.2 IBIDI microscopy assay

For multi-parameter analysis of a migrating cells' velocity, directionality and total distance travelled a 2D IBIDI µ-slide (IBIDI, #80301, Martinsried, Germany) system was utilised. Each slide was coated in 45µg/ml fibronectin (R&D Systems; diluted in PBS) overnight at room temperature. Then fibronectin coated slide was pre-warmed at 37°C for 1 hour before washing twice with ultra-pure MilliQ water. Basal cell migration was observed by completely filling the slide with media (phenol red free RPMI-1640 with 0.1% BSA; Figure 2.3.A). Whereas to observe chemotaxis, 18µl of chemokine CXCL11 (100nM; Peprotech, Table 2.3) was added to the top well of the IBIDI chamber (Figure 9.B). According to the manufacturer the chemokine will diffuse to give a gradient with an approximate concentration of 33nM reaching the cells (Figure 2.3.C). SEB activated T lymphocytes were washed three times in RPMI media and then re-suspended in chemotaxis media to give a concentration of 10 million cells/ml. 5µl of the cell

suspension (total of 50 cells) was pipetted into the slide observation platform (Figure 2.3A & B). The IBIDI μ -slide was then placed in an environmental chamber heated to 37°C and left for 15 minutes to allow the chemokine gradient to develop. An area containing at least 25 cells was selected upon the cell platform. A time series of images (Zeiss LSM 510 META microscope) were captured with a recording every 15 seconds for 15 minutes to allow a movie of cell migration to be generated. A total of 20 cells were tracked per condition using ImageJ software, the Manual Tracker plug-in (Fabrice Cordelieres) and analysed using the ImageJ chemotaxis tool (Gerhard Trapp and Elias Horn).

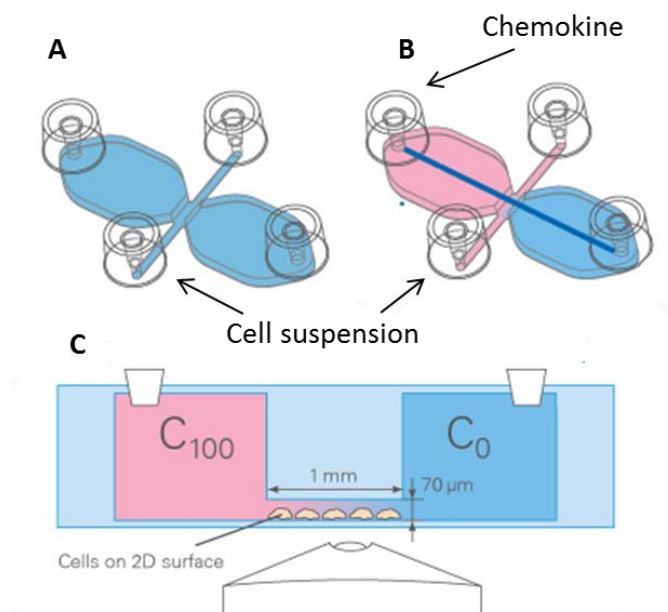


Figure 2.3 IBIDI μ slide for measuring *in vitro* cell migration. **A.** Top down view of IBIDI μ slide assessing basal migration with arrow indicating location to pipette cell suspension onto the central observation platform, blue colour indicates chemotaxis buffer without chemokine. **B.** Top down view of an IBIDI μ slide with arrow to indicating where chemokine and cells were added, pink colour represents the presence of chemokine. A chemokine gradient should develop across the slide indicated by the blue line **C.** Side view of an IBIDI μ slide showing a chemokine gradient between C_0 - C_{100} across the observation platform. Images taken from (Image taken from <http://.com/xtproducts/en/ibidi-Labware/Channel-Slides/m-Slide-Chemotaxis-2D>)

2.8 Confocal microscopy

1 million cells were stimulated as required and fixed in BD Fixation and Permeabilization (PERM) solution (which contains formaldehyde; BD Biosciences) for 30 minutes at 4°C. Cells were washed twice via centrifugation at 250g and re-suspension in BD PERM/Wash solution (containing sodium azide and saponin; BD Biosciences) and then incubated with 0.02% (v:v) anti-phospho ERM antibody (Cell Signalling Technology #3141) in BD PERM/Wash solution overnight at 4°C. Cells were washed twice in BD PERM/Wash solution, re-suspended in 0.01% (v:v) Goat anti-rabbit Fluorescein isothiocyanate (FITC) conjugated secondary antibody diluted in PERM/Wash solution overnight at 4°C. Cells were then washed twice in PERM solution, re-suspended in 20µl PERM solution and cells pipetted into Mowiol to adhere coverslips to slides then allowed to set overnight. Cells were visualized using a Zeiss LSM 510 Meta confocal microscope using Plan-Apochromat 63x/1.4 Oil DIC objectives. Cells were excited by 488nm light and fluorescence emission was collected at 520nm with a band pass filter of $530 \pm 15\text{nm}$. Images were collected at 2 x zoom.

2.9 Measurement of intracellular calcium

SEB-activated T lymphocytes were re-suspended in HBSS (Gibco) supplemented with 25mM HEPES and 0.1% BSA, pH 7.4 at 4 million cells per ml and loaded with 10µM cell permeant Fluo-4 acetoxymethyl (AM) [Invitrogen #F14201] for 45 minutes at 37°C, cells were protected from light. Fluo-4 is a calcium indicator which fluoresces upon binding to Ca^{2+} . Attaching Fluo-4 to an acetoxymethyl ester derivative allows the dye to pass through the cell membrane where it becomes cleaved by non-specific esterases and becomes trapped within the cell. Following incubation, cells were washed twice in Ca^{2+} free HBSS to remove any unloaded dye and density adjusted to either 1 million or 5 million cells per ml. Extracellular Ca^{2+} was adjusted to 1mM using CaCl_2 solution. Cells were loaded into a black flat bottomed 96 well plate at 100µl per well at allowed to rest at 37°C for 30 minutes. Cells were treated as stated in the figure legends and then plate was loaded into FluoStar Optima plate reader. Fluo-4 fluorescence was measured every 15s by exciting the cells at 485 nm and collecting emitted fluorescence at 520 nm. 20 cycles after starting the recording, stated concentrations of UCHT-1, CXCL11 or H_2O_2 were added and recording temporally paused, compounds were manually added into corresponding wells and recording restarted. 1µM ionomycin (Sigma #I-24222), an effective Ca^{2+} inophore, was added to the cells to ensure

sufficient loading of Fluo-4. Each compound response was normalised to its respective ionomycin response.

2.10 Measurement of reactive oxygen species generation

SEB activated T lymphocytes were re-suspended in supplement free RPMI-1640 and labelled with 10µM 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA, Invitrogen, Paisley, UK) at room temperature protected from light for 45 minutes. Following incubation cells were washed twice into PBS to removed unloaded dye and re-suspended at 1 million cells per ml. 100µl of cell suspension was placed in each well of a pre-warmed black bottom 96 well plate and allowed to rest at 37°C for 30 minutes. Cells were treated with stated concentrations of reagents for 30 minutes before endpoint recordings were taken on the FluoStar Optima plate reader at excitation/emission 490/515nm.

2.11 Adhesion assay

A flat bottomed 96 well plate was coated with either 10µg/ml recombinant human fibronectin (R & D systems #1918-FN) or 10µg/ml recombinant human ICAM-1 (R & D systems #ADP4-500) overnight. Plate was washed twice with pre-warmed PBS, then un-supplemented RPMI media (without phenol red). SEB activated cells were washed twice into un-supplemented media and treated with the stated concentrations of compounds for 30 minutes. Cells were then added to the 96 well plate and stimulated with UCHT-1 (10µg/ml). The plate was sealed and incubated for 30 minutes at 37°C to allow cells to adhere. The plate was then inverted for 15 minutes after which seal was removed whilst the plate was still inverted, all media and non-adherent cells removed from plate and wells washed gently with PBS. Then adherent cells quantified using the MTT assay, where cells are treated with MTT mix (RPMI, 10% FBS and 500µg/ml MTT) for 3h and then lysed in 100µl DMSO. Absorbance was read using a FluoStar Optima plate reader at 540nm

2.12 Hypoxic chamber experiments

The hypoxic chamber was equilibrated with either 5% O₂, 5% CO₂, 90% N₂ for 1 hour at 37°C. Cells were washed three times in plain media resuspended at 3.2 million per ml and then incubated in either standard cell culture conditions or in hypoxia for 1 hour prior to their migration being assessed using the methods describe for the Neuroprobe migration assay (Section 2.7.1). The migration assays were carried out in either hypoxic chamber or in standard cell culture conditions for the course of the experiment. Cells were also incubated in the hypoxic chamber for three hours prior to the evaluation of CXCR3 expression (2.6.1).

2.13 Data analysis

Data was normalised as described in the figure legends. Values are presented as mean \pm standard error of the mean (SEM) and n represents the total number of donors or individual repeats of each study. Statistical analysis was undertaken using GraphPad Prism software. Data was analysed using a One Way ANOVA followed by a Dunnett's post-test to determine significant difference as compared to control or when there were two independent variables a two-way ANOVA with Bonferroni post-test was used Differences were considered significant when * P<0.05, **P<0.01 ***P<0.001.

2.14 Pharmacological compounds

Inhibitors	Diluent	Stock	Supplier & Catalogue number	Target
Catalase from bovine liver	H ₂ O	Powder	Sigma-Aldrich, Dorset UK #C1345	H ₂ O ₂ consumption
H ₂ O ₂ Solution	RPMI	30%(w:v)	Sigma-Aldrich #216763	H ₂ O ₂
DPI (Diphenyleneiodonium chloride)	DMSO	50mM	Sigma-Aldrich #D2926	Flavin protein inhibitors
MnTBAP chloride	PBS	Powder	Abcam # ab141496	SOD dismutase mimetic
3 α -aminocholestane	Ethanol	10mM	Echelon # B-0341	SHIP-1 inhibition
AQX-1	DMSO	10 mM	Gift from Aquinox Pharmaceuticals	SHIP-1 activation
AQX-1125	DMSO	10 mM	Gift from GSK	SHIP-1 activation
CXCL11	PBS	10 μ M	Peprtech #300-46	CXCR3 activation
CXCL12	PBS	10 μ M	Peprtech # 300-28A	CXCR4 activation
CXCL10	PBS	10 μ M	Peprtech # 300-12	CXCR3 activation
PP2	DMSO	10 mM	Tocris Bioscience, Bristol UK #1407	Src kinase inhibitor
Piceatannol	DMSO	10 mM	Tocris Bioscience, Bristol UK #1554	Syk kinase inhibitor
U-73343	DMSO	10 mM	Tocris Bioscience, Bristol UK # 4133	Inactive PKC inhibitor
U-73122	DMSO	10 mM	Tocris Bioscience, Bristol UK #1268	Active PKC inhibitor
LY249002	DMSO	10 mM	SelleckChem, Suffolk UK #S1105	PanPI3K inhibitor
ZSTK474	DMSO	10 mM	SelleckChem, Suffolk UK # S1072	PanPI3K inhibitor
AS252424	DMSO	10 mM	SelleckChem, Suffolk UK # S2671	PI3K γ inhibitor
IC87114	DMSO	10 mM	SelleckChem, Suffolk UK # S1268	PI3K δ inhibitor

Table 2.3 List of pharmacological manipulators employed in this study.

CHAPTER 3: Effect of pharmacological targeting of SHIP-1 upon human T lymphocyte biology

3.1 Rationale

PI3K signalling is involved in numerous cellular functions, such as growth, proliferation, migration and cytokine production (Fruman *et al.*, 2009; So *et al.*, 2012b; Okkenhaug, 2013) and is crucial for the normal functioning of the immune system. However, over-active PI3K signalling is observed in chronic inflammation, immune-mediated disorders as well as cancer (Yuan *et al.*, 2008; Ghigo *et al.*, 2010; Marwick *et al.*, 2010; Suarez-Fueyo *et al.*, 2011). The use of PI3K inhibitors to treat inflammatory disorders has to date been disappointing (Blunt *et al.*, 2012a). This has led to the search for alternative targets with which to selectively modulate PI3K activity in leukocytes. One such target, the lipid phosphatase SHIP-1, has particular promise due to its restricted expression in cells of hematopoietic lineage, limiting the activity of SHIP-1 targeted drugs specifically to the immune system. SHIP-1 de-phosphorylates PI(3,4,5)P₃, (the major product of receptor stimulated PI3K), at the D-5 position (Damen *et al.*, 1996; Lioubin *et al.*, 1996) leading to its degradation to PI(3,4)P₂. Activation of SHIP-1's phosphatase activity would therefore be predicted to decrease PI(3,4,5)P₃ levels, thus mimicking the effects of a PI3K inhibitor (Blunt *et al.*, 2012b). Thus, pharmacological SHIP-1 activation has been proposed as a potential therapeutic for use in diseases with over-active PI3K signalling.

Little is known about SHIP-1 in primary human T lymphocytes; to date the majority of work investigating SHIP-1 has been undertaken using genetic mouse models or shRNA SHIP-1 targeting, both of which have indicated pleiotropic roles for SHIP-1 in T lymphocyte biology. However, SHIP-1 silencing approaches are likely to disrupt adaptor/scaffolding roles of SHIP-1 (*i.e.* properties of SHIP-1 which are unique from its catalytic ability to negatively regulate PI3K). Therefore, we sought to determine the effect of pharmacological targeting the catalytic domain of SHIP-1, which should maintain the phosphatase-independent functions of SHIP-1. SHIP-1 activators are not commercially available; here a small molecule allosteric SHIP-1 activator, which is closely related to AQX1125 (henceforth referred to as AQX1) was supplied by Aquinox Pharmaceuticals for research purposes only. The SHIP-1 activator, AQX1 will be directly compared to inhibitor, 3-aminocholestane (3AC) to allow side by side evaluation of the effect of SHIP-1 activation and inhibition upon T lymphocyte biology. Overall, the potential of SHIP-1 activation as a putative treatment of T lymphocyte driven pathologies will be assessed.

3.1.1 Aims

This chapter aims to determine the consequences of SHIP-1 manipulation on human T lymphocyte biology. An allosteric SHIP-1 activator, which is closely related to AQX1125 (henceforth referred to as AQX1) was synthesized and supplied by Aquinox Pharmaceuticals for research purposes only (Stenton *et al.*, 2013a) and SHIP-1 inhibitor, 3-aminocholestane (Echelon Biosciences) will be used to:

- establish the functional roles of SHIP-1 in human T lymphocyte viability and TCR activation including investigating downstream Ca^{2+} elevation, proliferation and adhesion.
- assess the functional importance of SHIP-1 as a regulator of basal and chemotactic migration, chemokine signalling, polarisation and cytoskeletal protein rearrangement.

Overall, we will evaluate the potential of SHIP-1 as a novel therapeutic route to influence T lymphocyte driven inflammatory and autoimmune diseases.

3.2 SHIP-1 modulators alter recombinant SHIP-1 catalytic activity

SHIP-1 is a lipid phosphatase which is responsible for de-phosphorylating PI(3,4,5)P₃ to generate PI(3,4)P₂ and free phosphate. *In vitro* the phosphate product generated by SHIP-1 can be measured using malachite green molybdate; a chemical which reacts with free organic phosphate to form molybdophosphoric acid which absorbs light at 650nm. As such, malachite green molybdate can be used to determine the catalytic activity of SHIP-1.

Here, malachite green molybdate has been used to verify that free phosphate is generated when recombinant SHIP-1 is incubated with a surrogate *in vitro* substrate, inositol 1,3,4,5-tetrakisphosphate (IP₄) (Damen *et al.*, 1996) for 30 minutes at 37°C. Firstly, a significant increased the absorbance at 650nm was observed when IP₄ was incubated with recombinant SHIP-1 alone (Figure 3.1). This confirmed that the recombinant SHIP-1 was de-phosphorylating the *in vitro* substrate.

After validating the assay, it was necessary to verify the respective activity of both pharmacological manipulators of SHIP-1. The SHIP-1 activator used in this study is closely related to AQX-1125 and henceforth referred to as AQX1. AQX1 was synthesized and supplied Aquinox Pharmaceuticals for research purposes only (Stenton *et al.*, 2013b). The activity of AQX-1125, which was provided by GlaxoSmithKline was also validated. Both SHIP-1 activators (1mM) significantly enhanced the catalytic activity of SHIP-1, whilst lower concentrations of AQX1 had no effect upon catalytic activity of recombinant SHIP-1 (Figure 1 A). The SHIP-1 inhibitor, 3 α -aminocholestane (3AC; Echelon Biosciences) induced concentration dependent reduction in SHIP-1 catalytic activity, which was significant at all concentrations above 100 μ M where catalytic activity was reduced by 31.3 \pm 0.1% (Figure 1 B).

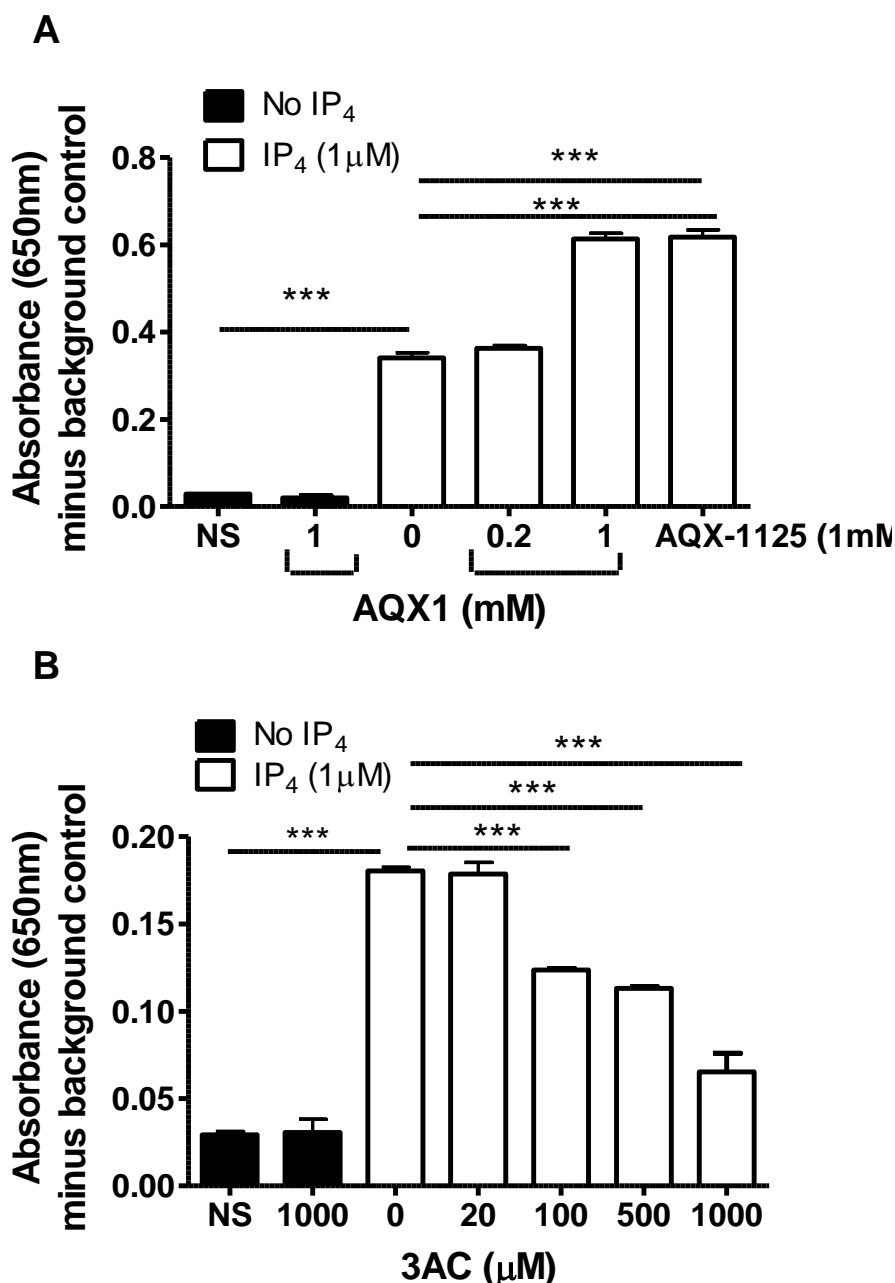


Figure 3.1 SHIP-1 modulators alter SHIP-1 catalytic activity. 1µg/ml recombinant human SHIP-1 (full length) was treated with vehicle control (DMSO [A] & ethanol [B]) or stated concentrations of (A) SHIP-1 activators or (B) SHIP-1 inhibitor for 30 minutes at 37°C in a 96 well plate. Inositol(1,3,4,5) tetrakisphosphate (IP₄) (100µM) was added for 30 minutes at 37°C. 100µl malachite green solution was added to each well, left for 15 minutes at room temperature and absorbance read at 650nm. The background absorbance of H₂O was subtracted from each well. Note that there is no change in absorbance in recombinant SHIP-1 alone or recombinant SHIP-1 treated with compound in the absence of IP₄, which is labelled no substrate (NS) and shown in black. Data are three independent experiments undertaken in duplicate. Statistical analysis was performed by a one-way ANOVA with a Dunnett's multiple comparisons post-test where ***p<0.001 relative to control.

3.3 SHIP-1 modulation has no effect upon SEB-activated T lymphocyte viability

PI3K signalling has known roles in cell growth and survival (Krasilnikov, 2000), which has led to interest in the role of SHIP-1 in cell survival. SHIP-1 has been shown to elicit a role in survival of mucosal and malignant T cells (Brooks *et al.*, 2010), with SHIP-1 inhibition preventing extrinsic cell death of mucosal T cells via the CD95/Fas/Caspase8 pathway (Park *et al.*, 2014). Therefore the effect of SHIP-1 modulators upon cellular viability of primary peripheral T lymphocytes was examined. To this end, MTT assays and Annexin V/Propidium iodide staining was utilized.

3.3.1 SHIP-1 modulators had no effect upon the MTT assay

Firstly, SEB-activated T lymphocytes were treated with increasing concentrations of SHIP-1 modulators and the MTT assay, a commonly used colorimetric enzyme assay, was used to assess cell viability. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced by NAD(P)H dependent cellular oxidoreductase enzymes to formazan which has a purple colour and absorbs light at 550nm. Although PI3K signalling is important in cell viability, 24hr treatment with AQX1 and 3AC had no significant effect upon cell viability as assessed by the MTT assay at any of the concentrations studied (Figure 3.2). This suggested that functional effects of AQX1 and 3AC shown in this chapter are not due to the compounds altering cellular viability.

3.3.2 SHIP-1 modulators had no effect upon Annexin V or PI staining

The impact of SHIP-1 modulators upon cellular apoptosis or necrosis was also examined using annexin V and propidium iodide (PI) staining. Apoptotic cell death is a tightly controlled process whereby cells are destroyed without leaking their contents into the extracellular space (Elmore, 2007). A key early step in apoptosis is a change in the lipid membrane structure, whereby phosphatidylserine (PS) which are normally held on the inner leaflet are presented on the outer leaflet of the cell membrane to aid phagocytosis. Externalised PS can be measured by binding of a fluorescently tagged form of Annexin V and quantified by flow cytometry. SEB-activated T lymphocytes were treated with stated concentrations of AQX1 and 3AC for 3 hours, there was no effect upon Annexin V fluorescence at any of the concentrations studied and thus they did not evoke cell apoptosis (Figure 3.3).

Necrotic cell death occurs in pathological conditions and results in un-restrained “bursting” of cells to release their intracellular content. PI uptake is an early marker for cellular necrosis, which emits fluorescence at 617nm when bound to nucleic acids. Positive control 10% Triton X, a detergent, significantly induced necrotic cells death. However, neither AQX1 nor 3AC had any effect upon T lymphocyte necrosis (Figure 3.3). This confirmed that observations obtained with AQX1 or 3AC were not due to them altering cellular viability.

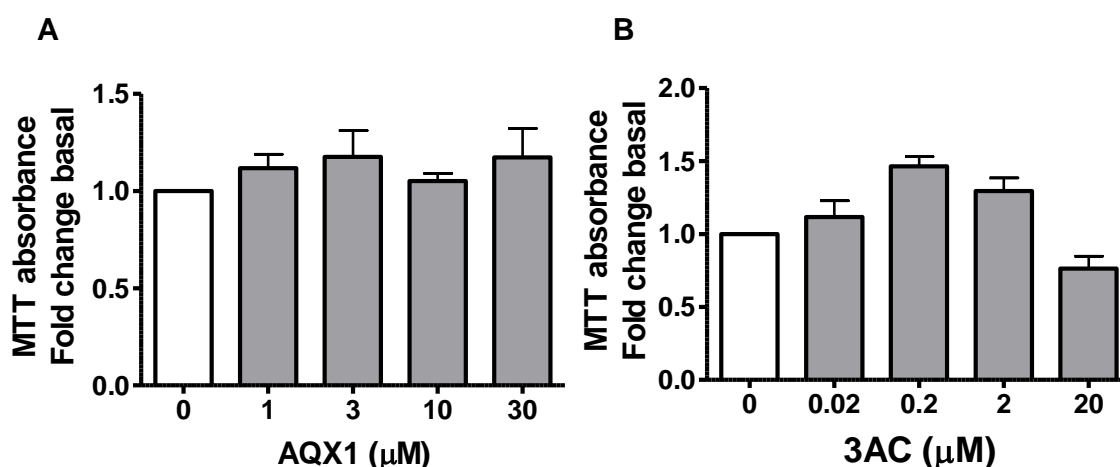


Figure 3.2 SHIP-1 modulators had no effect upon SEB-activated T lymphocyte viability. 1 million SEB-activated T lymphocytes (8-12 days post isolation) were treated with increasing concentrations of (A) AQX1 or (B) 3AC for 24 hours. Cells viability was assessed using the MTT assay. Vehicle control is indicated with the white bars and is (A) DMSO and (B) ethanol. Data are mean \pm SEM from three independent donors. Statistical significance was determined for treatment versus control using test one-way ANOVA indicating no significant difference between groups (A $p=0.6544$, B $p=0.09$).

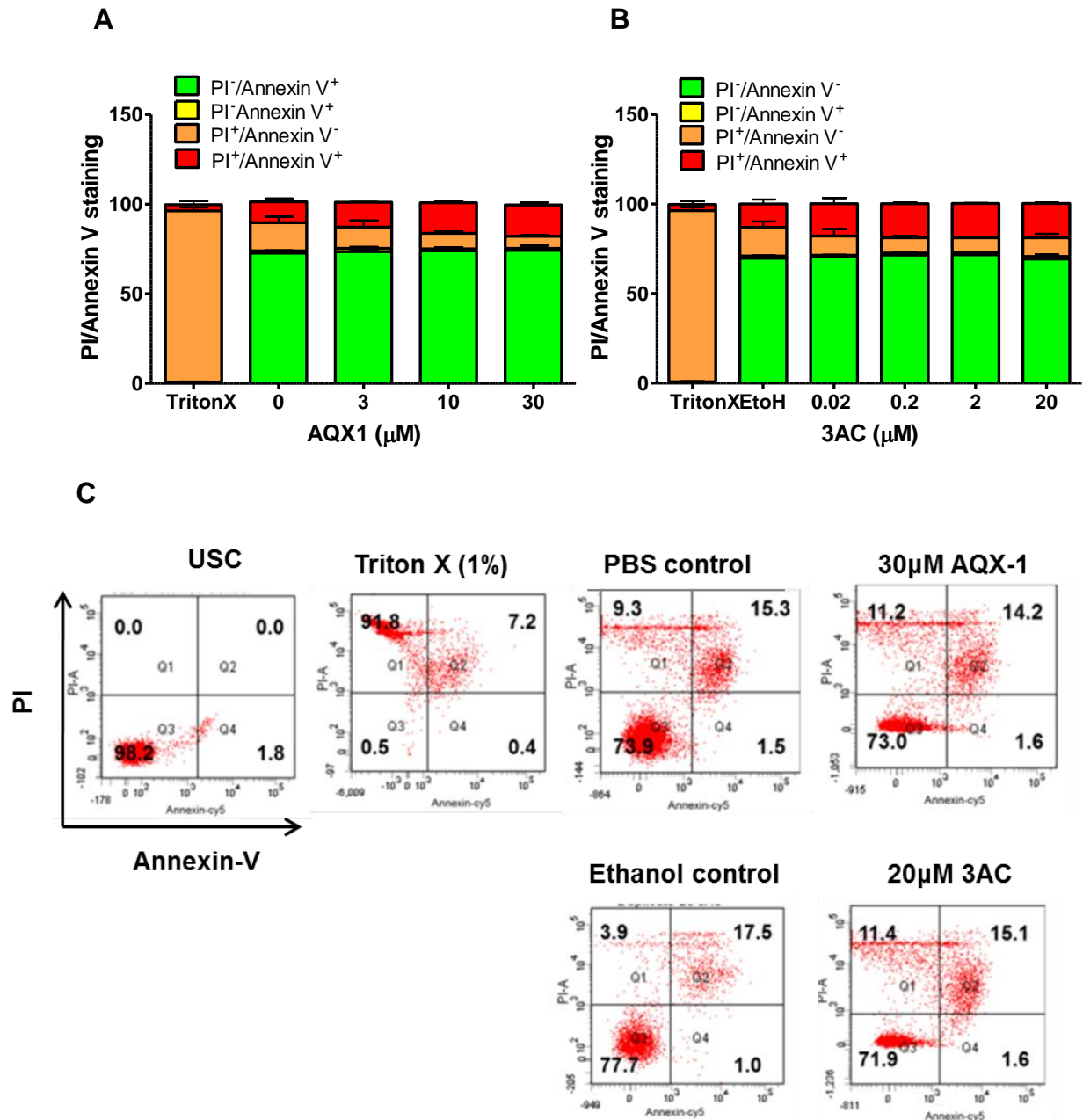


Figure 3.3. SHIP-1 modulators had no effect upon PI & Annexin V staining in SEB-activated T lymphocytes. 1 million SEB-activated T lymphocytes (8-12 days post isolation) were treated with the stated concentrations of (A) AQX1 or (B) 3AC, plus 1% TRITON-X as a positive control for 3 hours. T lymphocyte viability was established by staining cells with annexin V for 20 minutes at 4°C. Then, 2.5 $\mu\text{g}/\text{ml}$ propidium iodide (PI) was added to the cells and fluorescence emission read immediately using flow cytometry and laser excitation of 488nm (Cy5) and 617nm (PI). Viable cells are represented in green, annexin V+ cells in yellow, orange bars are PI+, and double positive cells are red. The bar chart shows the mean \pm SEM from three donors. (C) Representative FACS plots from one donor.

3.4 Effect of SHIP-1 modulation upon TCR induced responses

Ligation of the TCR receptor induces the activation of the PI3K/Akt signalling cascade which is crucial for driving lymphocyte clonal expansion and proliferation (Okkenhaug *et al.*, 2006). SHIP-1 has previously been observed to be recruited to the surface membrane upon CD3/CD28 ligation (Edmunds *et al.*, 1999), implicating a possible role of SHIP-1 in the regulation of TCR/CD3 signalling.

3.4.1 SHIP-1 modulation inhibits TCR/CD3 stimulated Akt phosphorylation

SEB T lymphocytes were pre-treated with stated concentrations of SHIP-1 modulators for 30 minutes. Cells were then stimulated with anti-CD3 antibody (UCHT1) for 5 minutes. Cells were fixed, permeabilised and levels of Akt phosphorylation determined by flow cytometry. The SHIP-1 activator AQX1 significantly inhibited UCHT1 stimulated Akt phosphorylation at 10 and 30 μ M AQX1 (Figure 3.4 A). Interestingly, the SHIP-1 inhibitor 3AC also caused a significant reduction in UCHT-1 stimulated Akt phosphorylation (Figure 3.4 B).

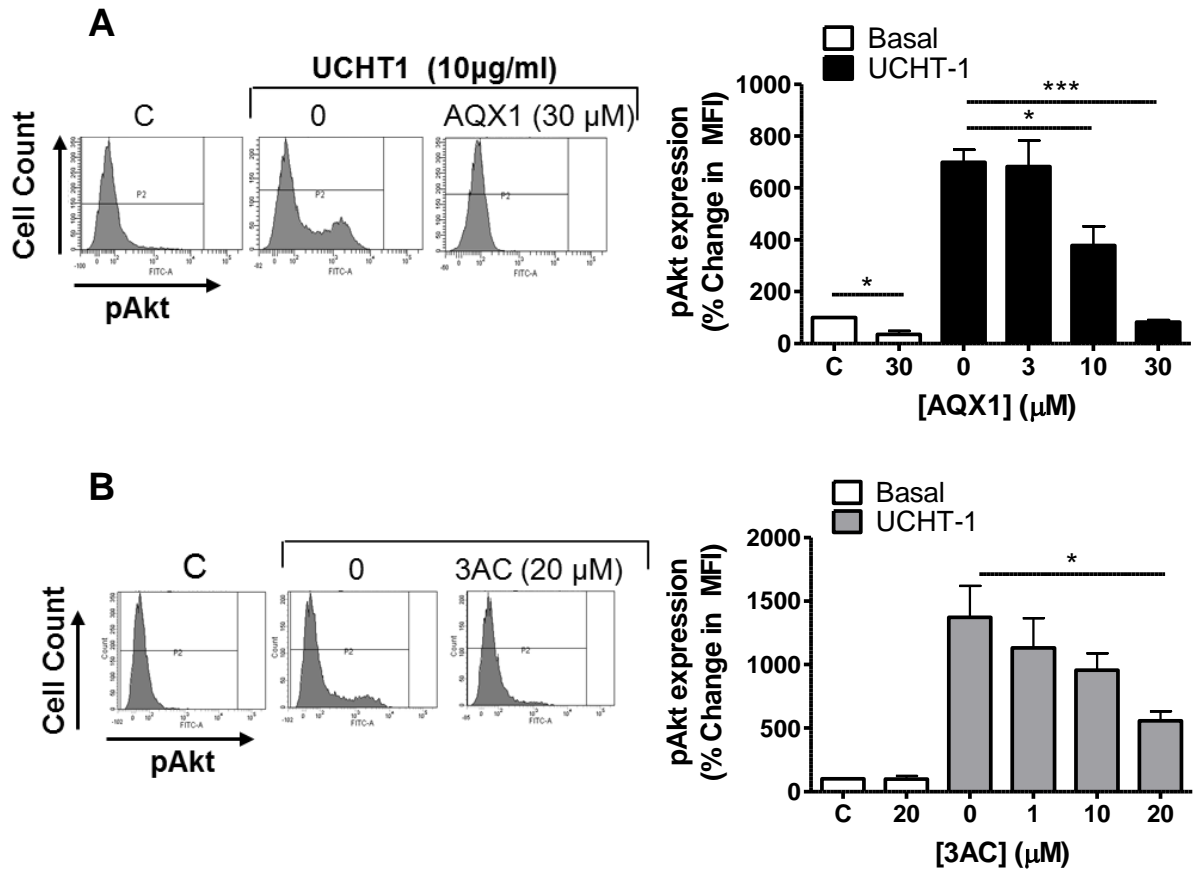


Figure 3.4 SHIP-1 modulation inhibits Akt phosphorylation in SEB-activated T lymphocytes. 1 million SEB-activated T lymphocytes (8-12 days post-isolation) were washed three times in serum free media and treated with stated concentrations of (A) AQX1 or (B) 3AC for 30 minutes. Cells were washed in ice cold PBS, then fixed using BD fixation reagent for 20 minutes. Cells were washed once and then treated with BD permeabilization reagent for 15 minutes. Cells were then incubated pAkt antibody for 30 minutes, washed twice in PERM reagent then incubated with FITC conjugated secondary antibody. Mean fluorescence intensity per 10,000 cells was measured using flow cytometry. Data are (left panel) representative FACs plot and (right panel) mean \pm SEM minus IgG control, normalised to untreated control from three independent donors. Statistical analysis was determined by one way-ANOVA with Dunnett's post-hoc test where * p <0.05 or *** p <0.001 relative to control.

3.4.2 SHIP-1 modulation has no effect upon TCR/CD3 induced Ca^{2+} release

An important signal produced upon TCR ligation is the elevation of intracellular Ca^{2+} . PLC γ 1 recruitment activation via SFKs and SYK signalling produces inositol 1,4,5-trisphosphate (IP3), which upon binding to the IP3 receptor releases Ca^{2+} from intracellular stores and induces subsequent Ca^{2+} dependent Ca^{2+} entry through membrane channels. These signals are vital in Ca^{2+} dependent gene transcription, such as nuclear factor of activated T cells (NFAT) and MAPK mediated activating protein-1 (AP-1) (Negulescu *et al.*, 1994) and initiate the expression of important genes involved in TCR-induced proliferation, differentiation and viability. Thus, Ca^{2+} mobilisation was evaluated to determine the effect of SHIP-1 targeting pharmacological tools affected TCR signalling potential.

SEB-activated T lymphocytes were loaded with a Ca^{2+} sensitive dye Fluo-4, and fluorescence measured over time using a plate reader to measure intracellular free Ca^{2+} . Initially, Ca^{2+} elevation following exposure to anti-CD3 antibody UCHT-1 was validated. UCHT-1 induced a concentration dependent and sustained increase in intracellular Ca^{2+} (Figure 3.5 A). The ionophore ionomycin was used as a loading control to indicate maximal Fluo-4 signal. To test whether SHIP-1 modulation could affect the UCHT-1 elevated intracellular Ca^{2+} , SEB T lymphocytes were pre-treated with either the SHIP-1 activator AQX1 or SHIP-1 inhibitor 3AC. AQX1 appeared to partially suppress the initial peak Ca^{2+} elevation but this observation was not significant across the three donors (Figure 3.5 B). 3AC also had no effect upon the UCHT-1-induced Ca^{2+} signal (Figure 3.5 C).

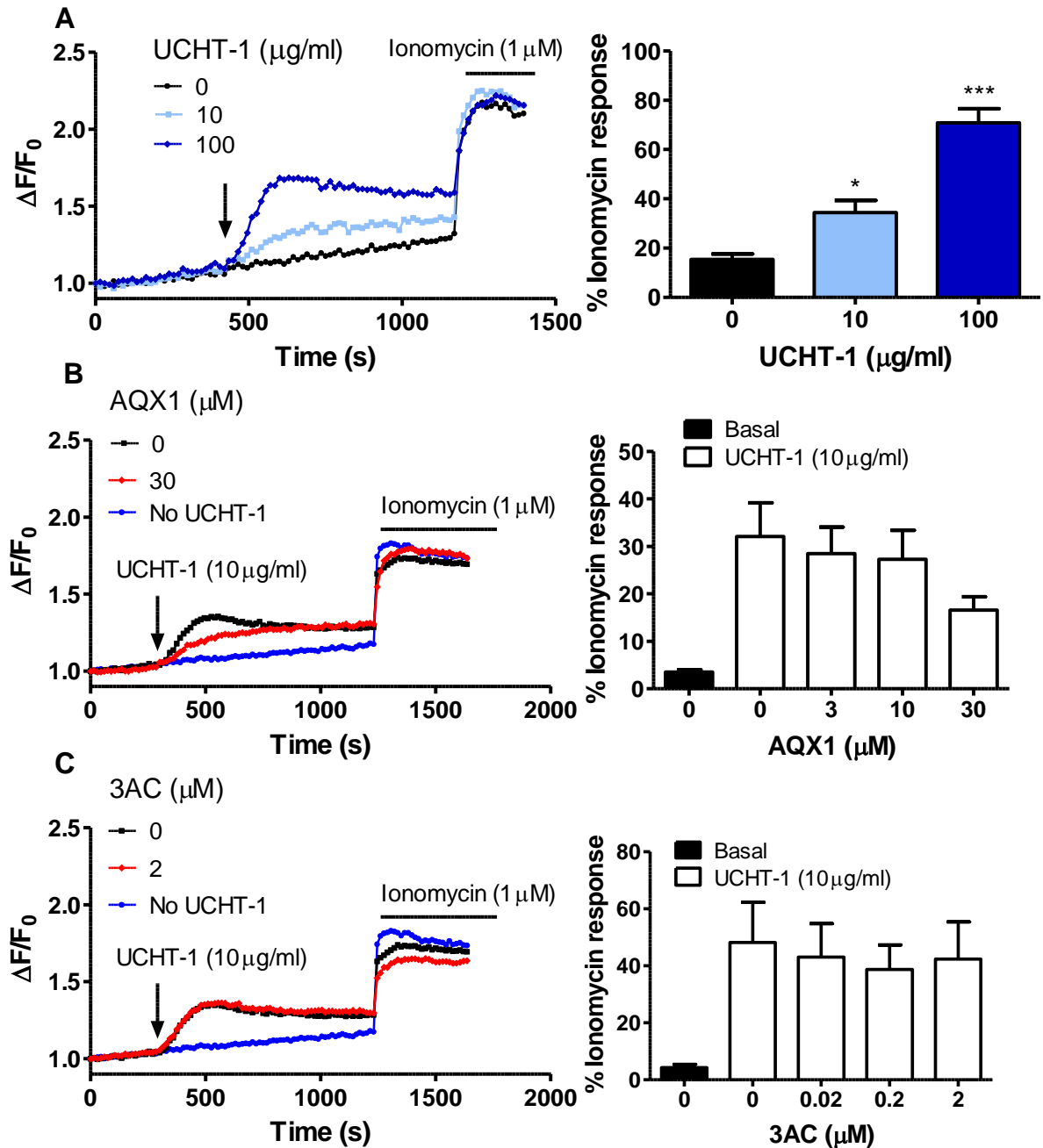


Figure 3.5 SHIP-1 modulators had no effect upon TCR agonist UCHT-1-induced intracellular Ca^{2+} release in SEB-activated T lymphocytes. SEB-activated T lymphocytes (8-12 days post isolation) were washed into PBS and loaded for 45 minutes with $1\text{ }\mu\text{M}$ Fluo-4. Cells were washed 3 times and re-suspended at 1 million cells per ml in Ca^{2+} free-HBSS and extracellular Ca^{2+} adjusted to 1 mM . Cells were plated in a black 96 well plate and either (A) allowed to rest or treated with stated concentrations of (B) AQX1 or (C) 3AC for 30 minutes. Stated concentrations of UCHT-1 were added after a baseline was established and $1\text{ }\mu\text{M}$ ionomycin was used as a loading control. Left panel is a kinetic trace indicating change in fluorescence from basal ($\Delta F/F_0$) from one representative donor. The right panel is the peak change in fluorescence generated by UCHT-1 as a percentage of the peak change generated by ionomycin and is mean \pm SEM from averaged duplicates of three independent donors. One-way ANOVA followed by Dunnett's post-test was used to determine statistical significance where $*p < 0.05$ and $***p < 0.001$ relative to control.

3.4.3 SHIP-1 modulation significantly impairs TCR driven proliferation

Given the crucial role of PI3K/Akt in cell proliferation (Okkenhaug *et al.*, 2006; Okkenhaug, 2013) and the ability of SHIP-1 compounds to affect TCR-induced Akt phosphorylation, the effect of SHIP-1 modulation on TCR/CD3-mediated clonal expansion of human T lymphocytes was investigated. Cells were labelled with the fluorescent probe CFSE and flow cytometry used to track the dilution of the CFSE as the cells divide. CFSE binds covalently to intracellular amines and the probe splits equally into each daughter cell.

Naïve T lymphocytes were treated with stated concentrations of the SHIP-1 modulators for 30 minutes before stimulation with anti-CD3/CD28 coated Dynabeads and IL-2. Initial CFSE intensity was determined on the day of isolation. CFSE intensity was quantified four days post isolation with plots from control conditions showing four distinct peaks, indicating four generations of cell division (Figure 3.6). The SHIP-1 activator AQX1 increased the percentage of cells retained in generation 1, whereas fewer cells were seen to progress to generation 4 (Figure 3.6 A). This indicates that activation of SHIP-1 inhibited the ability of human CD4 lymphocytes to proliferate and progress through cell divisions upon TCR/CD3 stimulation. Surprisingly, the SHIP-1 inhibitor 3AC only inhibited proliferation at high concentrations. 20µM 3AC increased the percentage of cells retained in generation 1; whereas no cells were observed to progress to generation 4 (Figure 3.6 B). To fully understand whether this observation relates to an issue with toxicity additional concentrations of 3AC between 2 and 20µM should have been examined.

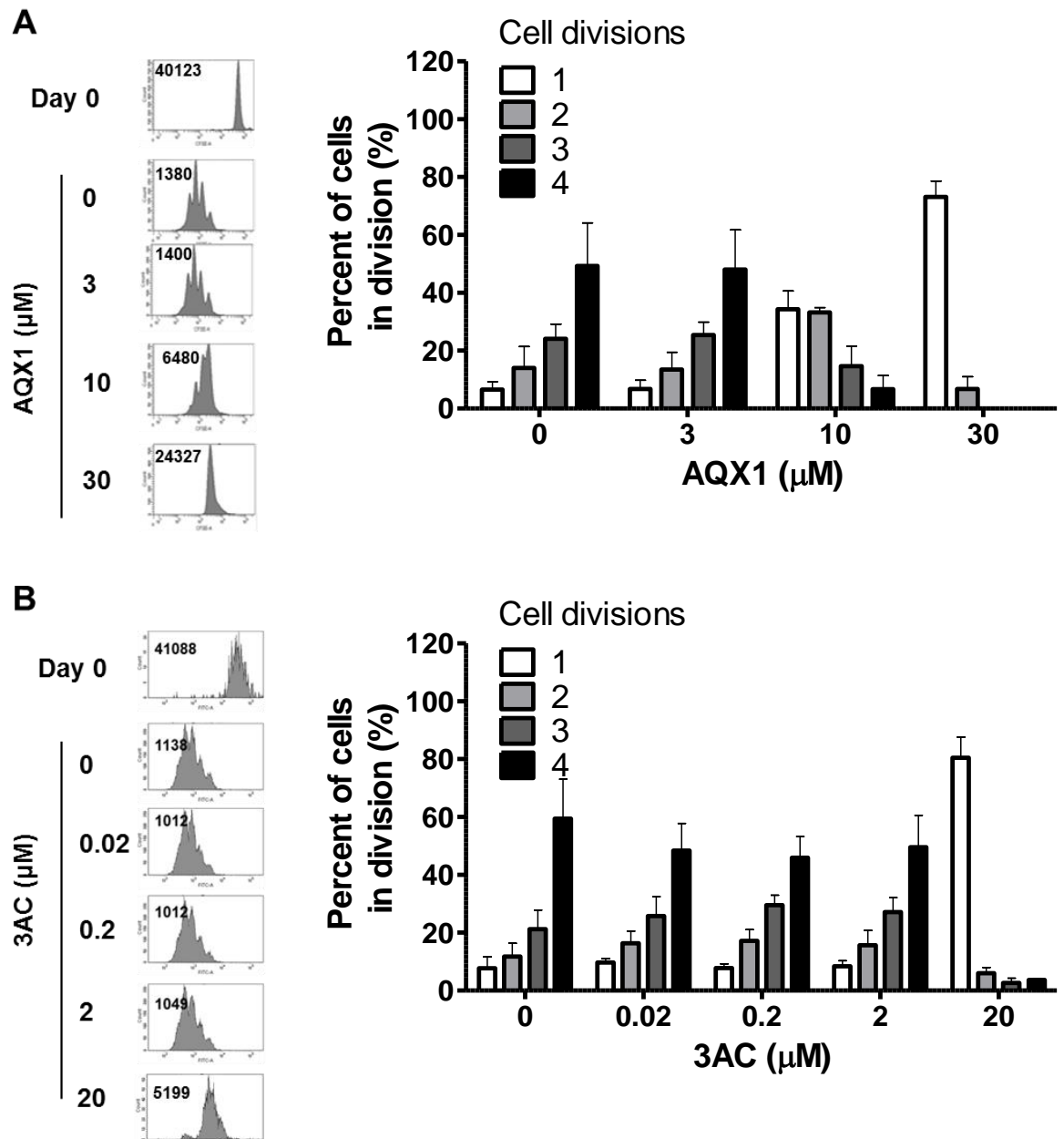


Figure 3.6 Activation of SHIP-1 inhibits TCR/CD3 mediated T lymphocyte proliferation. 1 million freshly isolated naïve CD4 cells were treated with increasing concentrations of (A) AQX1 or (B) 3AC for 30 minutes then stimulated with anti-CD3/anti-CD28 Ab-coated Dynabeads and IL-2 (36U/ml) for 4 days. Cell proliferation was assessed using CFSE staining and flow cytometry. Each panel shows a representative FACS plot. The numbers stated in the top left corner represent the mean fluorescence index of CFSE, and bar charts are the mean \pm SEM of the % of cells in each generation from three independent experiments in the absence or presence of indicated concentrations of AQX1 or 3AC.

3.4.4 SHIP-1 modulation impairs basal and TCR-induced adhesion of SEB T lymphocytes

Pharmacological modulation of SHIP-1 could also inhibit other functional outcomes of TCR activation. Ligation of the TCR is known to increase adhesion of lymphocytes (Simonson *et al.*, 2006), whilst the ability of lymphocytes to adhere to extracellular matrix components like fibronectin and adhesion molecules such as ICAM-1 is a crucial step in their extravasation (Stanley *et al.*, 2008; Nourshargh *et al.*, 2010), stabilising cellular contacts and facilitating accumulation of lymphocytes at sites of inflammation (Rodriguez *et al.*, 1992).

SEB-activated T lymphocytes were treated with increasing concentrations of SHIP-1 modulators for 30 minutes and their ability to adhere to either fibronectin or ICAM-1 was determined under basal and UCHT-1 stimulated conditions. UCHT-1 significantly enhanced the adherence to fibronectin by 5.6 ± 0.5 fold and ICAM-1 by 5.67 ± 1.1 fold. The SHIP-1 activator AQX1 abrogated both basal and TCR/CD3-induced adhesion of previously activated T lymphocytes to both fibronectin (Figure 3.7 A) and ICAM1 (Figure 3.7 B). Remarkably, the SHIP-1 inhibitor 3AC also abrogated basal and TCR/CD3-mediated adhesion to both fibronectin (Figure 3.7 C) and ICAM1 (Figure 3.7 D). This implies that a balance of PI(3,4,5)P3 and PI(3,4)P2 signalling is required for T lymphocyte adhesion. Interestingly, 3AC appears to have a more pronounced effect upon UCHT-1 induced adhesion, which suggests SHIP-1 induced PI(3,4)P2 signalling is required for TCR induced adhesion.

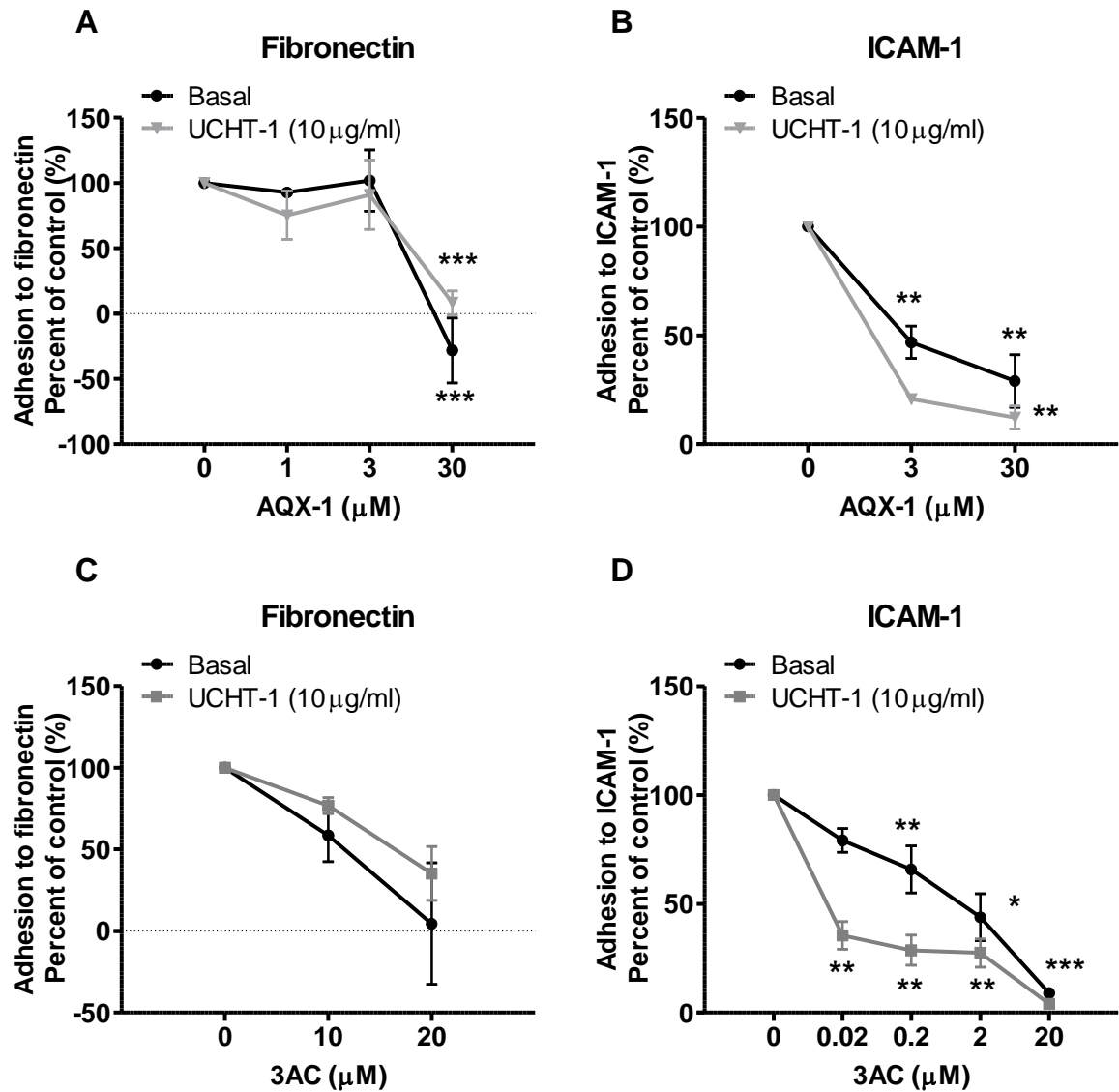


Figure 3.7 SHIP-1 modulation attenuates adhesion of SEB-activated T lymphocytes. 1 million SEB-activated T lymphocytes (8-12 days post-isolation) were treated with increasing concentrations of (A & B) SHIP-1 activator AQX1 or (C & D) SHIP-1 inhibitor 3AC for 30 minutes. Cells were allowed to adhere to either a (A & C) fibronectin (10 μ g/ml) or (B & D) ICAM-1 coated 96 well plate for 30 minutes. The plate was sealed and turned upside down for 15 minutes. Wells were washed gently to remove any un-adhered cells. Cells were scrapped and counted using FACSCanto flow cytometry. Data are represented as percent change from basal conditions and is mean \pm SEM of three independent experiments. Statistical significance was determined by one-way ANOVA with a Dunnett's multiple comparisons post-test where * p <0.05 ** p <0.01, *** p <0.001 as compared to control.

3.4.5 SHIP-1 has no effect upon integrin expression but alters the affinity state of LFA-1

In an attempt to unravel the mechanism underlying the decreased adhesion, the effect of SHIP-1 modulation upon the expression of the adhesion receptors CD11a and CD49d was examined. CD11a is the α L subunit which along with CD18 (β 2) forms the integrin dimer LFA-1 (α L β 2). LFA-1 binds to ICAM-1, present on leukocytes and the endothelium, to aid intercellular contacts and extravasation. CD49d is the α 4 subunit, which along with CD29/ β 1, forms the integrin dimer VLA-4 (α 4 β 1) (Larson *et al.*, 1990). VLA-4 is one of several integrins that can bind to the extracellular matrix protein fibronectin. Interestingly, neither the SHIP-1 activator AQX1 nor SHIP-1 inhibitor 3-AC had any effect on the expression of CD11a or CD49d (Figure 3.8 A & B).

However, LFA-1 exists in different states of affinity which determines the strength of ICAM1-LFA-1 interactions. The result of SHIP-1 modulation upon the affinity state of LFA-1 was examined using monoclonal antibodies (mAb) that recognize differentially exposed epitopes expressed upon activation. Following activation, LFA-1 extends, exposing an epitope that is recognised by mAb KIM127, located in the EGF-like domain 2 at the bend in the β 2 subunit (Robinson *et al.*, 1992; Beglova *et al.*, 2002). The extended KIM127+ LFA-1 adopts both closed and open conformations corresponding to intermediate- and high-affinity forms of LFA-1, respectively (Kinashi, 2006; Luo *et al.*, 2007). Interestingly, the SHIP-1 activator AQX1 significantly reduced the detection of KIM127+ LFA-1 expression, thus indicating a reduced amount of LFA-1 in its activated affinity state (Figure 3.8 C). Consistent with its effect on adhesion, SHIP-1 inhibitor 3AC also reduced detection of KIM127+ LFA-1 in previously activated T lymphocytes (Figure 3.8 D). This suggests that SHIP-1 modulates T lymphocyte adhesion to ICAM-1 by changing the conformation and affinity state of LFA-1.

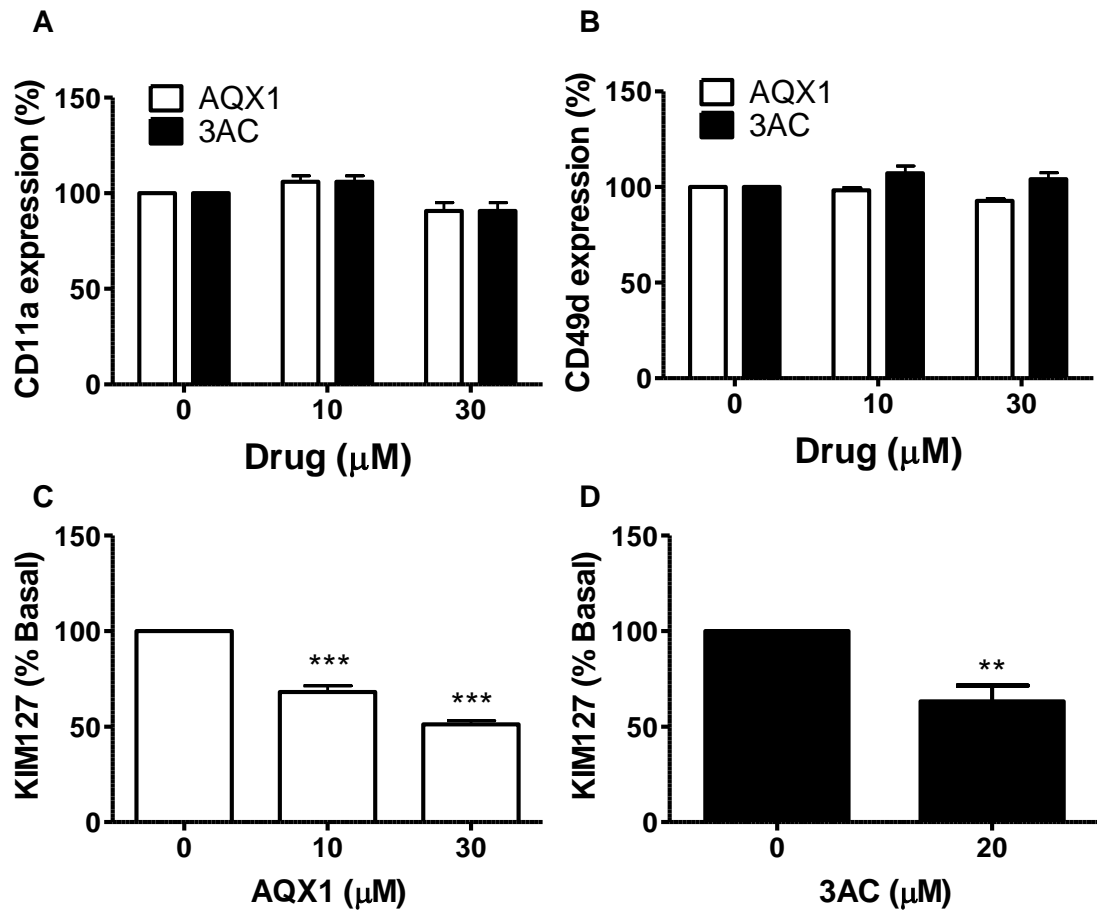


Figure 3.8 SHIP-1 modulation attenuates adhesion of T lymphocytes by reducing the affinity state of LFA-1. 1 million SEB-activated T lymphocytes (8-12 days post-isolation) were treated with either increasing concentrations of SHIP-1 activator AOX1 or SHIP-1 inhibitor 3AC for 30 minutes and then expression of surface receptors (A) CD11a and (B) CD49d was assessed using PE-conjugated antibodies. SEB-activated T lymphocytes were treated with increasing concentrations of (C) AOX1 or (D) 3AC for 30 minutes, blocked for 1 hr in 1% BSA and the stained with the primary antibody KIM127 (10 μ g/ml) for 30 minutes at 4°C and washed twice in PBS. Cells were then incubated for 30 minute with anti-mouse FITC conjugated antibody at 4°C, washed twice in PBS and analysed by FACSCanto flow cytometry. Data are represented as percent change from basal conditions and is mean \pm SEM of three independent experiments. Statistical significance was determined by one-way ANOVA with Dunnett's post-test where ** p <0.01, *** p <0.001 as compared to control.

3.5 Effect of SHIP-1 modulation upon CXCL11 driven responses

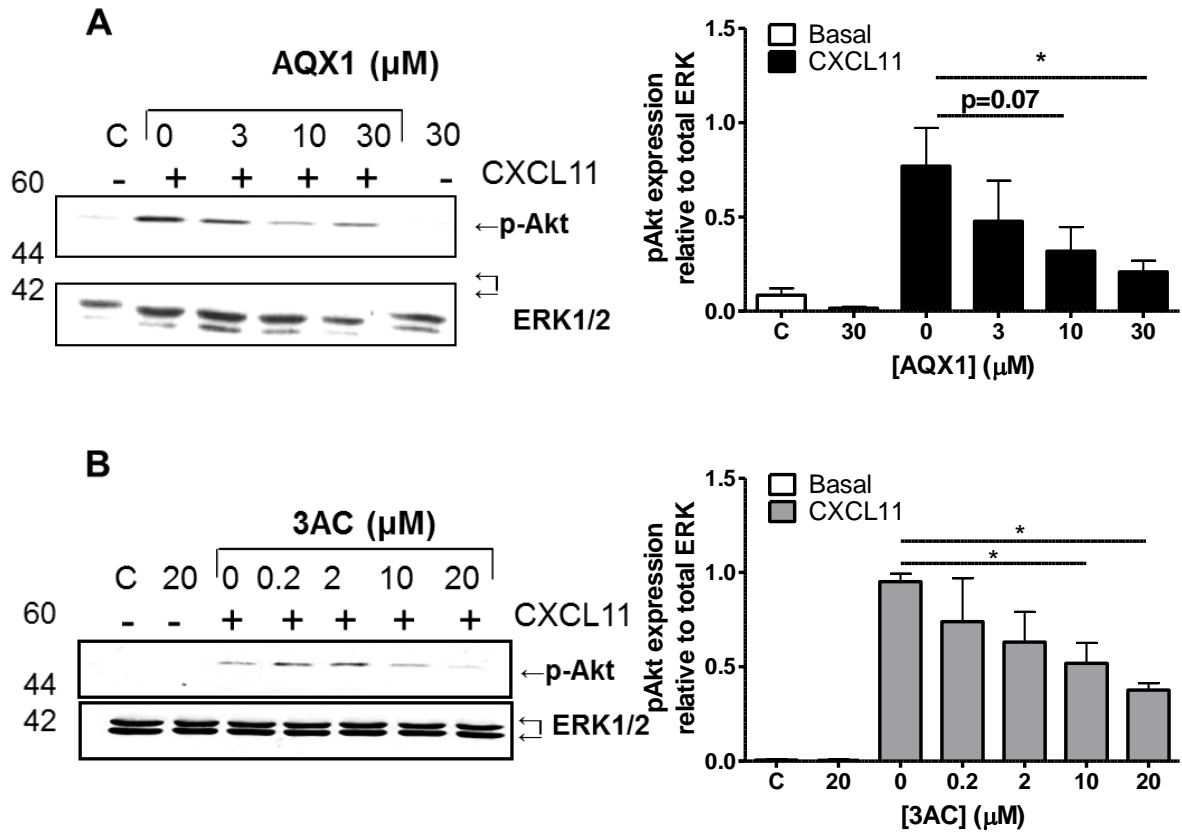
In addition to regulating PI3K signalling downstream of tyrosine kinase-coupled antigen receptors, SHIP-1 has previously been demonstrated to regulate PI3K-dependent signalling activated by G protein-coupled receptor (GPCRs), including CXCR3. Following antigen encounter, the chemokine receptor CXCR3 is up-regulated on T lymphocytes and is crucial for their recruitment into inflamed tissues (Marwick *et al.*, 2010). The role of SHIP-1 in CXCL11/CXCR3 signalling, migration and polarisation of T lymphocytes was therefore examined.

3.5.1 SHIP-1 modulation inhibits CXCL11-induced Akt phosphorylation in SEB T lymphocytes

Akt phosphorylation is used as an indirect measure of PI3K activity. SEB-activated T lymphocytes were pre-treated with SHIP-1 modulators for 30 minutes, stimulated with CXCL11 for 5 minutes and the effect of SHIP-1 modulation on Akt phosphorylation was determined by Western Blot. Importantly, SHIP-1 activator AQX1 caused a concentration dependent reduction in CXCL11 stimulated Akt phosphorylation (Figure 3.9 A). Similarly, SHIP-1 inhibitor 3AC also abrogated CXCL11-stimulated Akt phosphorylation (Figure 3.9 B), indicating that SHIP-1 modulation can alter CXCL11-induced signalling.

3.5.2 SHIP-1 modulation had no effect upon CXCL11 induced Ca^{2+} elevation

Another important signal produced upon CXCR3/CXCL11 stimulation is the elevation of intracellular Ca^{2+} concentration. Elevated intracellular Ca^{2+} is important in directed cell migration as local Ca^{2+} pulses near the leading edge of the cell activate myosin and to modulate focal adhesions (Tsai *et al.*, 2014). Therefore intracellular Ca^{2+} levels were measured to assess whether SHIP-1 modulation affected CXCR3/CXCL11 signalling potential. SEB-activated T lymphocytes were loaded with a Ca^{2+} sensitive reporter Fluo-4, and fluorescence was measured over time using a plate reader to measure intracellular Ca^{2+} . CXCL11-induced a transient concentration dependent, albeit modest, increase in intracellular Ca^{2+} (Figure 3.10 A). The number of cells in each well was increased to 500,000 to boost the signal (*i.e.* the total level of Ca^{2+} elevation) for the CXCL11 experiment. To test whether SHIP-1 modulation could affect the CXCL11 elevated intracellular Ca^{2+} , SEB T lymphocytes were pre-treated with either SHIP-1 activator AQX1 or inhibitor 3AC for 30 minutes. Neither compound had any effect upon CXCL11-induced Ca^{2+} signal (Figure 3.10 B & C).



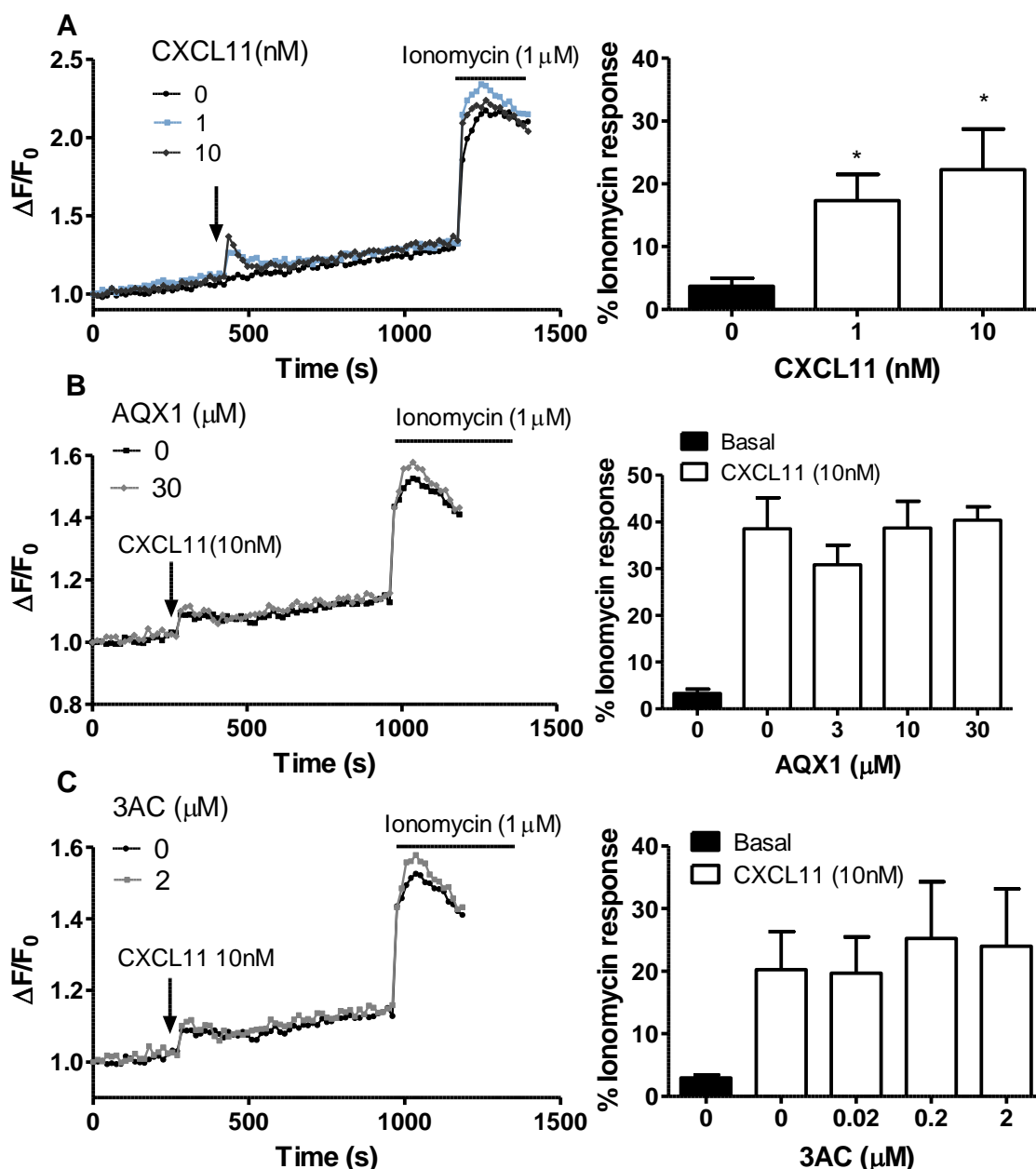


Figure 3.10 SHIP-1 modulators had no effect upon CXCL11-induced intracellular Ca^{2+} release in SEB-activated T lymphocytes. SEB-activated T lymphocytes (8-12 days post-isolation) were washed into PBS and loaded for 45 minutes with 1 μ M Fluo-4. Cells were washed 3 times and re-suspended at 5 million cells per ml in Ca^{2+} free-HBSS and extracellular Ca^{2+} adjusted to 1mM. Cells were plated in a black 96 well plate and either (A) allowed to rest or treated with increasing concentrations of (B) AQX1 or (C) 3AC for 30 minutes. Fluorescence was recorded over time using a FluoStar Optima plate reader at 37°C. Stated concentrations of CXCL11 were added after a baseline was established and 1 μ M ionomycin was used as a loading control. Left panel is a kinetic trace indicating change in fluorescence from basal ($\Delta F/F_0$) from one representative donor. The right panel is the peak change in fluorescence generated by UCHT-1 as a percentage of the peak change generated by ionomycin and is the mean \pm SEM from averaged duplicates of three independent donors. One-way ANOVA followed by Dunnett's post-test was used to determine significance where * $p < 0.05$ as compared to control.

3.5.3 SHIP-1 modulation attenuated both basal and CXCL11 directed migration in SEB-activated T lymphocytes

Consistent with the reduction in chemokine driven Akt signalling, SHIP-1 modulation altered basal and chemokine-induced lymphocyte migration. First, using a Neuroprobe plate-based assay that measured migration across a synthetic membrane, SHIP-1 activator AQX1 reduced basal and chemotactic migration with an IC_{50} of 9.9 μ M and 970nM, respectively. SHIP-1 inhibitor 3-AC reduced basal and chemotactic migration of previously activated T cells with an IC_{50} of 29nM and 4.3 μ M, respectively (Figures 3.11 A & B).

Second, migration was also assessed using the IBIDI μ -slide chemotaxis assay which allows tracking of individual cells across a fibronectin-coated surface (Figure 3.12 A & B). AQX1 and 3AC abrogated CXCL11 mediated migration as measured by accumulated distance and velocity. Using the IBIDI assay, a reduction in basal migration in the presence of AQX1 or 3AC was not observed. However, this assay has negligible basal migration compared to the NeuroProbe assay.

3.5.4 SHIP-1 modulation had no effect upon the surface expression of CXCR3 in SEB-activated T lymphocytes

As SHIP-1 modulation significantly impaired CXCL11 driven migration and signalling, its effect upon cognitive receptor CXCR3 expression was observed. A 30 minute treatment of SHIP-1 manipulators was used in an attempt to limit any transcriptional changes which would be unlikely to occur within this short timeframe, and instead focused upon determining the effect of SHIP-1 upon receptor trafficking. Neither AQX-1125 nor 3AC had any effect upon CXCR3 expression (Figure 3.13).

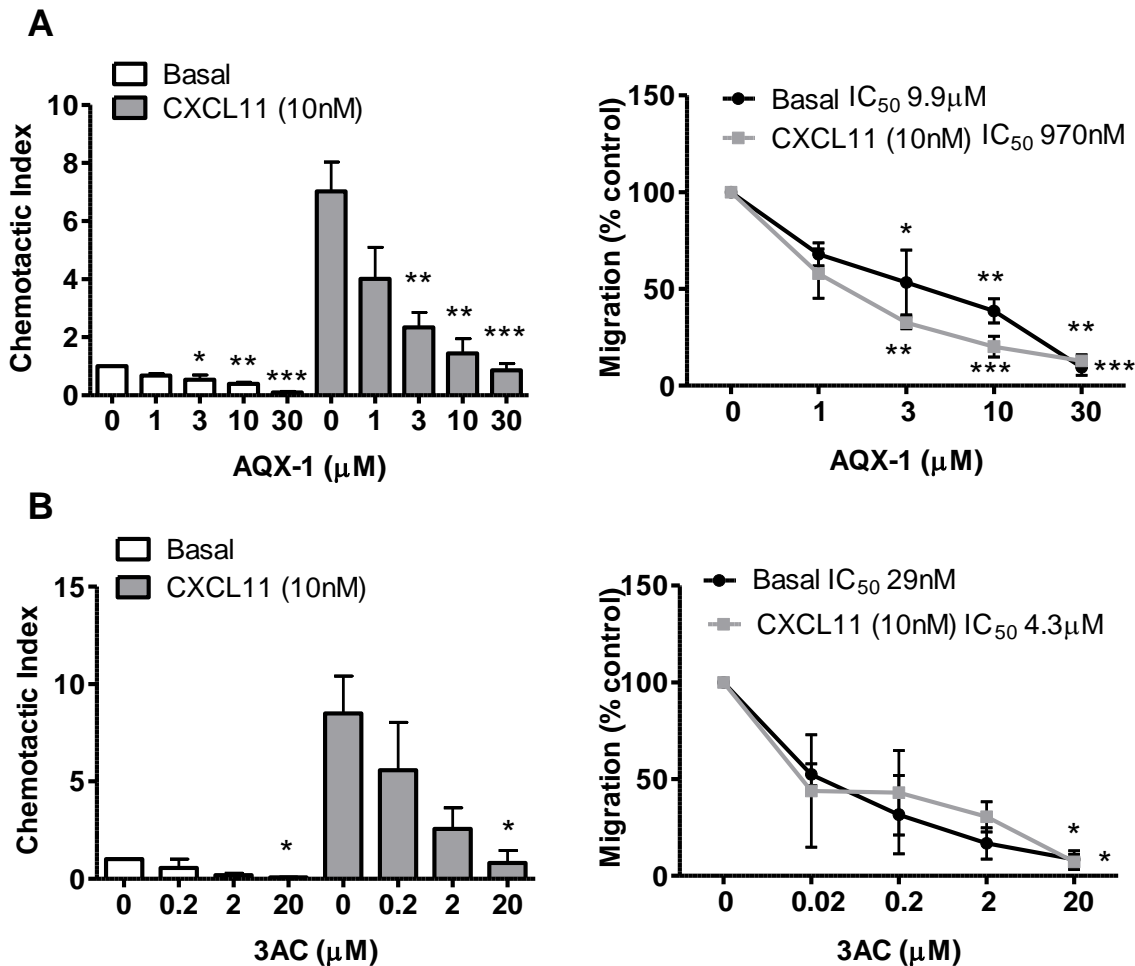


Figure 3.11 SHIP-1 modulation inhibits basal and chemokine stimulated migration in SEB-activated T lymphocytes. SEB-activated T lymphocytes (8-12 days post isolation) were washed in serum free media and resuspended at 3.2×10^6 cell/ml. Cells were pre-treated with increasing concentrations of (A) AQX1 or (B) 3AC 30 minutes at 37°C . The basal and chemokine CXCL11 (10nM)-induced migration of cells was then determined using the Neuroprobe assay. Left panel is expressed as chemotactic index where the number of cells migrated with each drug condition is divided by the vehicle control and the right panel is the percentage of vehicle control migration. Data are the mean \pm SEM of three independent donors. Right panel shows migration as a percent of vehicle control. Statistical significance was determined by one way ANOVA with a Dunnett's post-test where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to control.

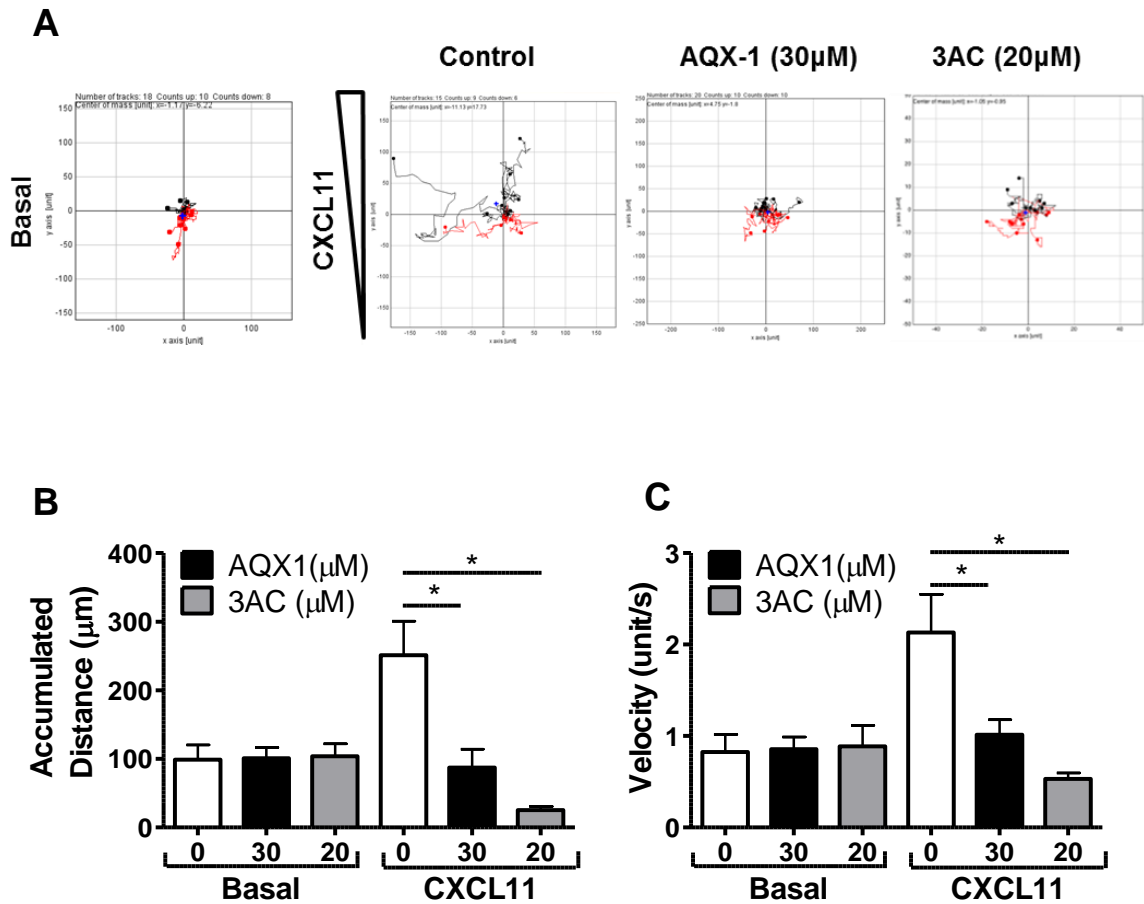


Figure 3.12 Chemotaxis on a fibronectin-coated surface is reduced by SHIP-1 modulation. SEB-activated T lymphocytes (8-12 days post-isolation) were washed and pre-treated with either vehicle, 30µM AQX1 or 20µM 3AC 30 minutes at 37°C. Migration of cells was observed using IBIDI µslides coated in 10µg/ml fibronectin. Cells were added to the slides and allowed to adhere for 15 minutes. Cells were imaged using the bright-field channel of a 40x objective on a confocal microscope. Time-lapse images were taken every 15s and cell migration stimulated either PBS or CXCL11 (100nM). Cell tracking was performed using the ImageJ Manuel tracking plugin. (A) Shows a representative ImageJ tracking plot from one donor. Data are the mean \pm SEM of three independent donors with (B) accumulated distance and (C) velocity. Right panel shows migration as a percent of vehicle control. Significance was determined by two-way ANOVA with Bonferroni's post-test where * $p < 0.05$ as compared to vehicle control.

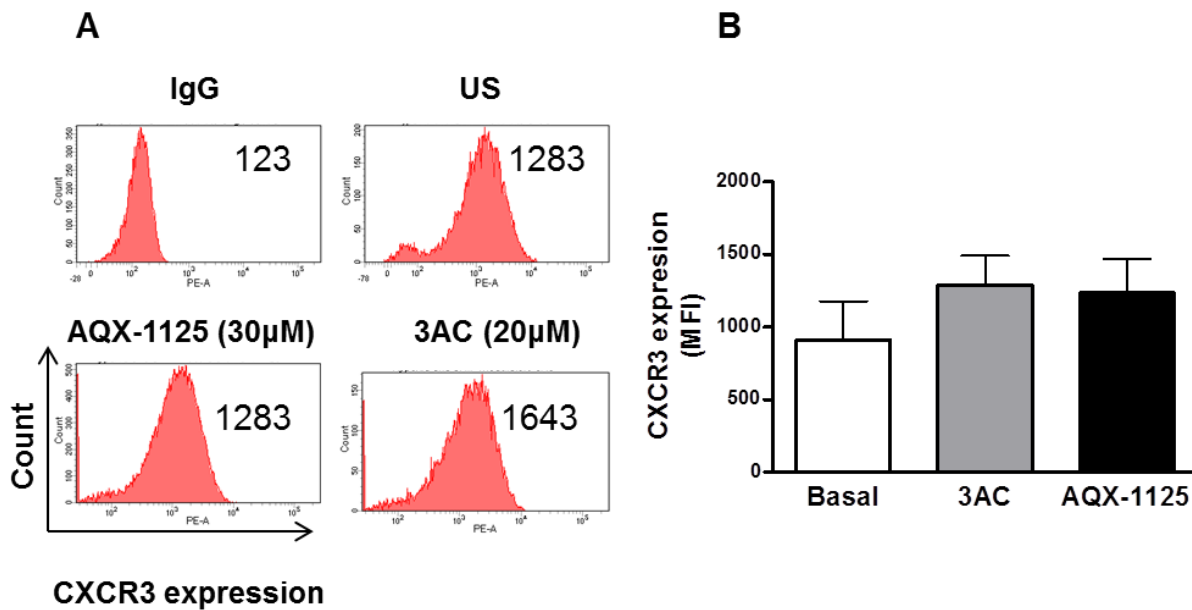


Figure 3.13 SHIP-1 modulation has no effect upon surface receptor expression of CXCR3 in SEB-activated T lymphocytes. 1 million SEB-activated T lymphocytes (8-12 days post-isolation) were washed three times in serum free media and treated with AQX1125 (30 μ M) or 3AC (20 μ M) for 30 minutes. Cells were washed in ice cold PBS, then blocked in 1% BSA for 30 minutes. Cells were then incubated in either IgG control antibody or Alexa-PE-conjugated CXCR3 antibody on ice for 30 minutes and washed twice in ice cold PBS. Mean fluorescence intensity per 10,000 cells was measured using flow cytometry. Data are (A) representative FACS plots with MFI in top right corner and (B) mean \pm SEM minus IgG control, normalised to untreated control from three independent donors. Statistical significance was determined by one way-ANOVA with Dunnett's post-test.

3.5.5 SHIP-1 modulation reduces chemokine dependent de-phosphorylation of Ezrin, radixin and moesin in SEB-activated T lymphocytes

Phosphorylation of ezrin, radixin and moesin (ERM) proteins, which link the actin cytoskeleton to the cell surface membrane, have previously been implicated in cell migration and adhesion (Arpin *et al.*, 2011). In T lymphocytes, phosphorylation of ERM proteins is rapidly reduced by chemokine stimulation (Brown *et al.*, 2003). In addition, silencing of SHIP-1 expression in T lymphocytes was found to cause the de-phosphorylation of ERM proteins (Harris *et al.*, 2011). The impact of pharmacological SHIP-1 activation or inhibition upon the phosphorylation of ERM proteins was therefore examined. Naïve T lymphocytes were treated with SHIP-1 activator AQX1, followed by visualisation of ERM phosphorylation by confocal microscopy. CXCR4 is highly expressed on naïve T lymphocytes and therefore the effect of CXCL12 on ERM proteins was examined. CXCL12 caused a significant de-phosphorylation of ERM proteins. Interestingly, AQX1 pre-treatment prevented CXCL12 dependent de-phosphorylation (Figure 3.14 A)

Consistently, immunoblots indicated that 3AC or AQX1 treatment enhanced the phosphorylation of ERM proteins in SEB-activated T lymphocytes under un-stimulated conditions, whilst exposure to chemokine CXCL11 (Figure 3.14 B and C) caused a de-phosphorylation of ERM proteins. However, both pharmacological activation and inhibition of SHIP-1, with AQX1 or 3AC respectively, prevented CXCL11 mediated ERM de-phosphorylation (Figure 3.14 B & C). 50nM calyculin A (CalyA), a potent serine/threonine phosphatase inhibitor prevents ERM dephosphorylation and was used as a positive control to indicate high ERM phosphorylation.

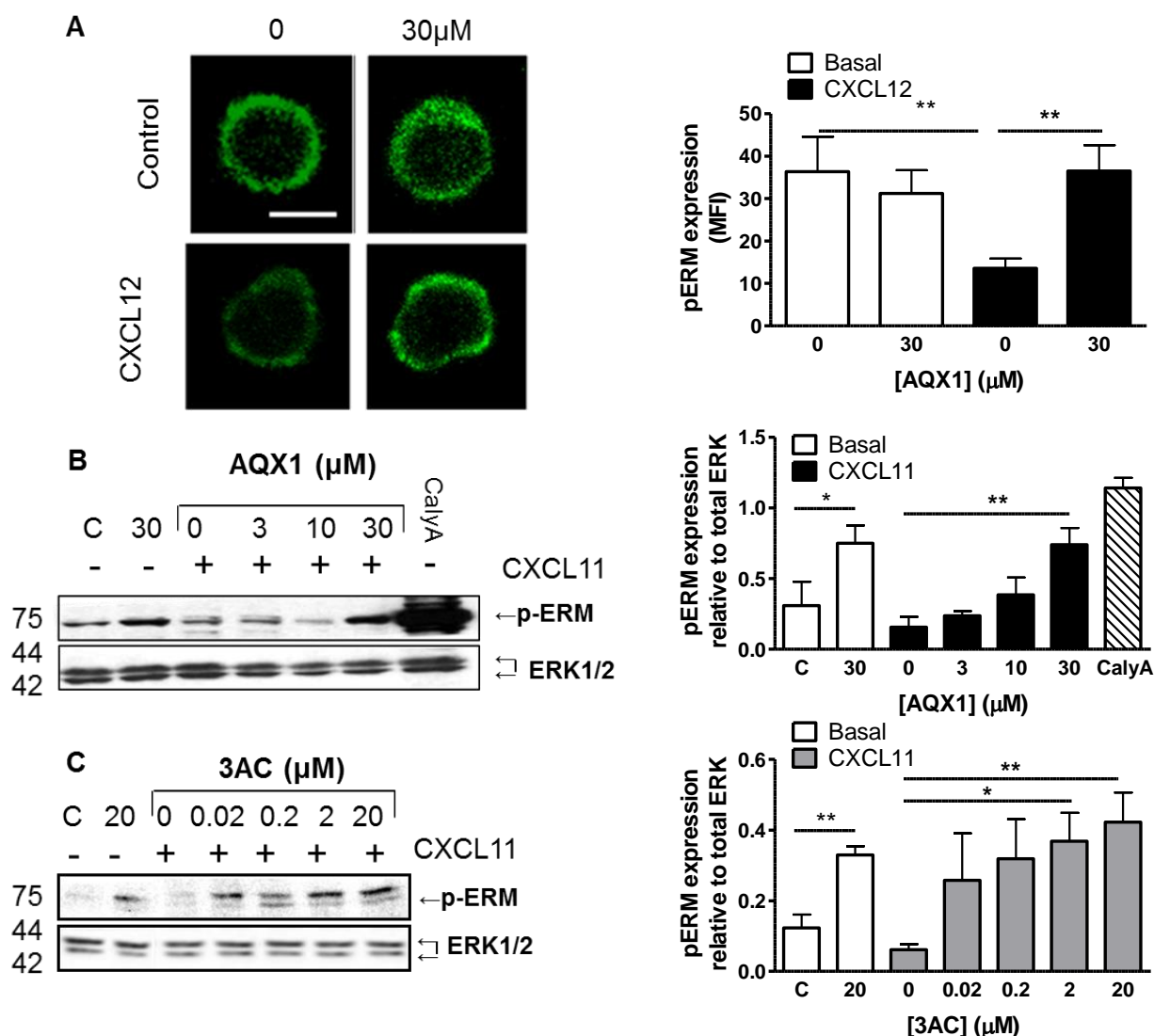


Figure 3.14 SHIP-1 modulation alters the phosphorylation state of ERM proteins in T lymphocytes. (A) Naïve T lymphocytes were treated with AQX1 (30µM) for 30 minutes the stimulated with CXCL12 (10nM) for 5 minutes. Cells were fixed in BD cytofix for 30minutes at 4°C and then exposed to anti-phospho-ERM overnight. Cells were stained with FITC conjugated secondary antibody and ERM phosphorylation detected by confocal microscopy. Left panel is representative of 6 images per treatment from 3 different donors. Scale bar = 10µm. Right panel is mean intensity of FITC fluorescence determined by using LSM 510 analysis software. SEB-activated T lymphocytes were incubated either with vehicle control (labelled C) or increasing concentrations of (B) AQX1 or (C) 3AC for 30 minutes prior to stimulation with CXCL11 for 5 minutes. 50nM Calyculin A (CalyA) was used as a positive control to indicate high ERM phosphorylation. Levels of phosphorylated ERM and total ERK were then assessed using Immunoblotting. Left panels are representative Westerns Blot from a single experiment, numbers on the left are kDa. The right panel are mean \pm SEM of phosphorylated ERM relative to total ERK, from three independent experiments. Statistical significance was determined by a one way ANOVA with Dunnett's post-test where * p <0.05 & ** p <0.01 as compared to control.

3.6 Chapter 3 Results Summary

- Compounds alter SHIP-1 catalytic activity in the malachite green assay
- Neither SHIP-1 activation or inhibition had any effect upon T lymphocyte viability
- TCR-induced Akt phosphorylation was inhibited by either SHIP-1 activation or inhibition
- Neither SHIP-1 activation nor inhibition had any effect upon TCR or CXCL11-induced Ca^{2+} elevation
- SHIP-1 activation inhibited CD3/CD28-induced proliferation of naïve T cells, but inhibition had no effect
- Basal and UCHT-1 stimulated adhesion to fibronectin and ICAM-1 were inhibited by SHIP-1 activation and inhibition
- Expression of CD11a and CD49d were unaffected by activation and inhibition of SHIP-1
- Activation and inhibition of SHIP-1 reduced the affinity state of LFA-1
- CXCL11 induce Akt phosphorylation was inhibited by SHIP-1 activation and inhibition
- SHIP-1 activation and inhibition attenuated basal and directed migration
- SHIP-1 activation and inhibition enhanced basal ERM phosphorylation and prevented CXCL11-induced de-phosphorylation
- Both activation and inhibition of SHIP-1 have the same functional consequences in SEB-T lymphocytes

3.7 Chapter 3 - Discussion

Validation of SHIP-1 modulators on malachite green absorbance

The SHIP-1 activator, AQX1 and inhibitor, 3 α -aminocholestane (3AC) were successfully validated to alter the catalytic activity SHIP-1 using the malachite green assay. Interestingly, the cell-based studies confirmed that SHIP-1 compounds were membrane permeant and that they manipulate cell responses at lower concentrations than in isolated protein conditions. Although the structure of AQX1 has not been disclosed due to a material transfer agreement with Aquinox Pharmaceuticals, in their hands AQX1 significantly enhanced the catalytic activity of recombinant SHIP-1 at 1 μ M, which is consistent with the concentrations of AQX1 that are active in our functional assays. The structure of AQX1 is related to the published compound AQX1125, which is currently in phase II clinical trials. AQX1125 (30 μ M) has been shown to be highly selective in a range of selectivity and specificity investigations (Stenton *et al.*, 2013a).

3AC was identified through a high-throughput fluorescence polarisation assay and shown to inhibit the catalytic activity of recombinant SHIP-1 by 50% at 10 μ M, but not affect effect PTEN or SHIP-2 at any of the studied concentrations (Brooks *et al.*, 2010). Intracellular modification or protein interactions might be required for full efficacy of SHIP-1 compounds in cell-based assays. To further quantify the compounds cellular potency upon SHIP-1 the cellular levels of PI(3,4,5)P3 and PI(3,4)P2 could have been investigated using integrated high performance liquid chromatography mass spectrometry.

SHIP-1s roles in cell survival, TCR signalling and proliferation

(a) Survival

Pharmacological activation and inhibition of SHIP-1 had no effect upon cell viability of primary human T lymphocytes isolated from the peripheral blood of healthy human donors. This implies that in peripheral T lymphocytes that altering the balance between level of phospholipid PI(3,4,5)P3 and PI(3,4)P2 signalling has no effect upon cell viability. This result is consistent with shRNA silencing of SHIP-1 in human T cells which also had no effect upon cell viability (Harris *et al.*, 2011) and in line with reports of T cell specific SHIP-1 knockout mice, where T cell numbers are unaffected

(Tarasenko *et al.*, 2007). Although it is in contrast to germline deletion of SHIP-1 in mice which leads to a decrease in the T lymphocyte population especially in the periphery (Helgason *et al.*, 1998; Kashiwada *et al.*, 2006). However lymphocyte abnormalities observed in SHIP-1 null mice could be a consequence of the severe myeloid pathology these mice present (*i.e.* interaction and cytokine release from other affected immune cells could lead a reduction in T lymphocytes).

Furthermore, recent evidence actually suggests a role for SHIP-1 product PI(3,4)P₂ in T lymphocyte survival. SHIP-1 expression was observed to prevent extrinsic cell death of mucosal T lymphocytes via the CD95, Fas, Capase 8 pathway (Park *et al.*, 2014). In addition, enhanced SHIP-1 activity and elevated PI(3,4)P₂ levels have been observed in several leukaemias (Luo *et al.*, 2003; Fukuda *et al.*, 2005; Brauer *et al.*, 2012); cells which exhibit high survival and proliferation. In some cases, PI(3,4)P₂ has been shown to promote Akt activation (Franke *et al.*, 1997) and tumorigenicity (Ivetac *et al.*, 2009). In addition, SHIP-1 inhibitor 3AC induces the apoptosis of human acute myeloid leukaemic cell lines (Brooks *et al.*, 2010) and inhibits multiple myeloma cell growth in a tumour xenograft model in mice (Fuhler *et al.*, 2012), suggesting SHIP-1 activity and thus PI(3,4)P₂ is required for the survival of these cells. As we did not observe any effect of 24hr treatment of 3AC on peripheral T lymphocyte survival, it could be interesting to further this work and investigate whether there is a differential requirement of SHIP-1 signalling in survival of malignant and peripheral T lymphocytes. If a differential requirement exists it could be beneficial in using SHIP-1 inhibitors to specifically kill malignant cells, sparing the normal adaptive T lymphocyte function required to combat opportunistic pathogens.

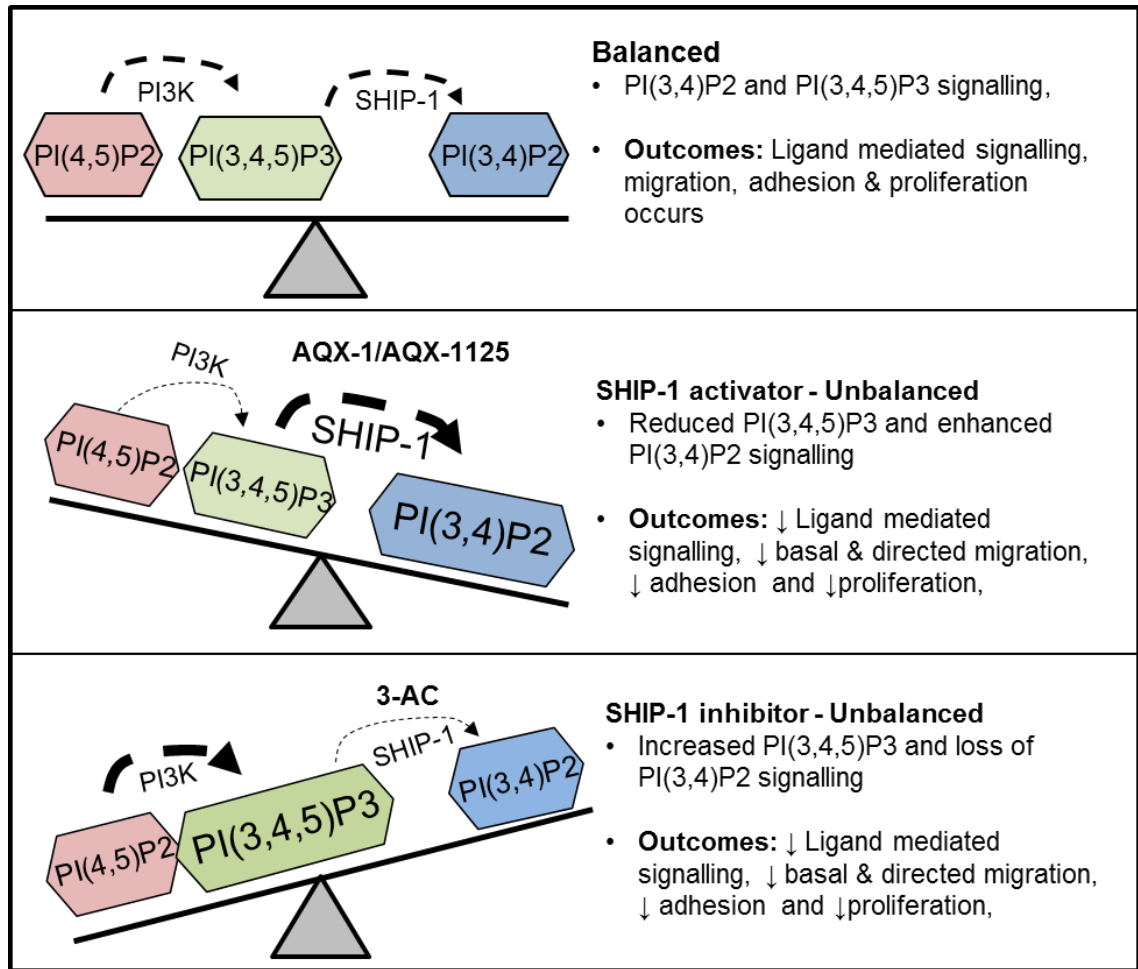
(b) TCR-dependent Akt phosphorylation

SHIP-1 is a major negative regulator downstream of cytokine (Lioubin *et al.*, 1996), Fc (Kiener *et al.*, 1997) and B cell receptors (Helgason *et al.*, 2000), but the evidence indicating SHIP-1 role in regulating TCR/CD3 signalling has proven more contradictory, with its exact biological functions poorly understood. Firstly, in agreement with SHIP-1 eliciting a role in TCR activation, interactions have been observed between both CD3 and the TCR ζ chain with the SH2 domain of SHIP-1 (Osborne *et al.*, 1996). Moreover, the stimulation of CD3 and CD28 caused both tyrosine phosphorylation and increased catalytic activity of SHIP-1, as well as a marked re-distribution of SHIP-1 from the cytosol to the surface membrane (Edmunds *et al.*, 1999). Restoration of membrane

localised SHIP-1 in Jurkats, leukemic T cells which lack SHIP-1 expression, reduces basal and TCR-induced PI3K signalling through Akt (Edmunds *et al.*, 1999) .

SHIP-1 activation and inhibition reduced TCR/CD3 mediated Akt phosphorylation and proliferation human peripheral T lymphocytes, validating the importance of SHIP-1 in TCR dependent signalling. This is consistent with the observation that shRNA silencing of SHIP-1 also inhibits T lymphocyte Akt phosphorylation and proliferation (Harris *et al.*, 2011). Moreover, AQX1125 has also been shown to reduce Akt phosphorylation in the leukemic T cell line MOLT-4 (Stenton *et al.*, 2013a). However, in contrast conditional silencing of SHIP-1 in T lymphocytes in mice was found to cause no differences from wild-type controls in terms of TCR-induced signalling or proliferation (Tarasenko *et al.*, 2007) suggesting SHIP-1 is un-important in TCR signalling. These conflicting results could be due to differences between human and mouse T lymphocytes and highlight the fact that there are often substantial differences between the immune systems of mice and humans. This study indicates the importance of studying the role of proteins in human cells, even if the assays available are somewhat limited compared to animal models.

It is important to note that the product of SHIP-1 is distinct from the phospholipid which is generated from the direct antagonism of PI3K signalling (*i.e.* PI(4,5)P2). PI(3,4,5)P3 and PI(3,4)P2 have both been shown to interact with the PH domain of Akt, with phosphorylation at Serine-473 in Akt dramatically reduced in the absence of PI(3,4)P2 (Franke *et al.*, 1997; Scheid *et al.*, 2002; Ma *et al.*, 2008; Kerr, 2011). It has been suggested that a balance of these phospholipids is required maximal Akt activation which may explain, in part, why both the activator and inhibitor of SHIP-1 abrogated Akt phosphorylation and inhibited proliferation of T lymphocytes (Figure 3.15).



3.15 Activation and inhibition of SHIP-1 leads to the same functional outcomes.

Under normal conditions, PI3K and SHIP-1 regulate the levels and localisation of PI(3,4,5)P3 and PI(3,4)P2 to co-ordinate the normal functions of T lymphocytes. The balance between PI(3,4,5)P3 and PI(3,4)P2 appears to be vital. Due to its abundance in basal cells PI(4,5)P2 is not believed to have a signalling role. Activation of the endogenous negative regulator of PI3K, SHIP-1 with pharmacological tools such as AQX1 leads a decrease in PI(3,4,5)P3 and an accumulation of PI(3,4)P2 and inhibition of T lymphocyte function. Similarly, inhibition of SHIP-1 with 3AC leads to downregulation of PI(3,4)P2 signalling and an accumulation of PI(3,4,5)P3 signalling, which also reduces T lymphocyte function.

PI(3,4)P2 has a prominent positive signalling role in cells (Franke *et al.*, 1997; Tiwari *et al.*, 2009; Brooks *et al.*, 2010) and is able to recruit and activate unique PH-domain containing proteins to PI(3,4,5)P3, including TAPP1/TAPP2 (Marshall *et al.*, 2002; Zhang *et al.*, 2009; Li *et al.*, 2013), lamellipodin (Lpd) (Krause *et al.*, 2004; Law *et al.*, 2013), and SNX9 (Posor *et al.*, 2013), indicating that instead of producing the same effect as PI3K inhibition, SHIP-1 activation could drive distinct signalling (Figure 3.16). PI3K inhibitors inhibit the accumulation of PI(3,4,5)P3 within cells, therefore conceptually they also eliminate PI(3,4)P2 dependent signalling, as SHIP-1 no-longer has a substrate to de-phosphorylate (Scheid *et al.*, 2002). It is interesting to compare our observations with SHIP-1 modulation to the literature on PI3K inhibition to determine the potential benefits of both methods in treating diseases of the immune system and this is touched upon in different sections in the discussion. TCR/CD8 mediated Akt activation has been shown to be dependent on PI3K regulatory subunit p85s and p110 δ in peripheral T cells (Okkenhaug *et al.*, 2006; Deane *et al.*, 2007; Garcon *et al.*, 2008). p110 δ -deficient mice T lymphocytes also exhibit deficient clonal expansion to CD4 T lymphocytes (Okkenhaug *et al.*, 2006). The p110 δ inhibitor IC87114 inhibits proliferation of both mouse and human T lymphocytes (Soond *et al.*, 2010). Thus Inhibition of p110 δ appears to exhibit a similar phenotype to SHIP-1 modulation in human T lymphocytes.

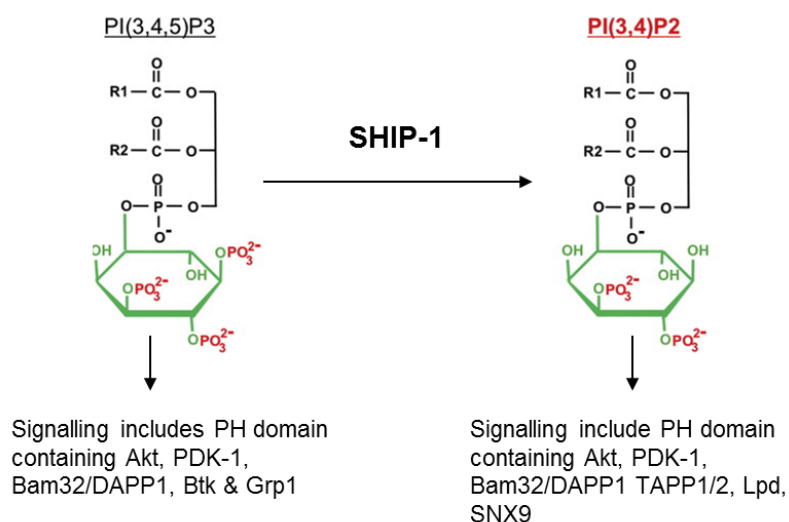


Figure 3.16 Chemical structures and downstream signalling proteins of phosphoinositides. Phosphatidylinositol (PI) consists of a phosphatidic acid backbone, linked via a phosphate group to an inositol ring head group (green). R1 and R2 denote fatty acid groups. PI can be phosphorylated at various hydroxyl groups of the inositol ring. Phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3) and PI(3,4)P phosphatidylinositol-3,4-biphosphate (PI(3,4)P2) are illustrated alongside the PH domain containing proteins which can recruited to each phospholipid sub-type.

It is also interestingly to point out that although PI(3,4,5)P3 is necessary for full activation of Akt, Akt can be activated and phosphorylated by serine/threonine kinase, inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKBKE) independently of a PI3K/PH domain mediated mechanism (Guo *et al.*, 2011). Thus PI3K inhibition or SHIP-1 modulation may not fully silence Akt signalling. Direct inhibition of Akt has also been explored as a putative cancer treatment (Hers *et al.*, 2011; Tan *et al.*, 2011). However, paradoxically direct inhibition of Akt could actually increase PI3K-dependent activation of its other effectors. This is because Akt activation leads to increased mTORC1 activity, which operates a well-known negative feedback mechanism (Guo *et al.*, 2011). When mTORC1 is active, the PI3K-Akt pathway is suppressed, whereas if mTORC1 is inhibited (for instance, with rapamycin), PI3K-Akt signalling can actually be enhanced (Guertin *et al.*, 2009), thus modulating SHIP-1 appears to be a better method of manipulating Akt activation and downstream signalling than direct Akt inhibition.

(c) Proliferation

Actin rearrangement is known to be important in cell division with dysregulation causing failure of centrosome separation and spindle assembly in the S phase of the cell cycle (Bendris *et al.*, 2015). SHIP-1 modulation impairs ERM cytoskeletal protein phosphorylation and rearrangement and SHIP-1 silencing inhibits actin rearrangement (Hogan *et al.*, 2004; Harris *et al.*, 2011) which could underlie why SHIP-1 modulation affects T lymphocyte proliferation. Proliferation of peripheral T lymphocytes may also be reliant upon suppressed TCR-induced Akt phosphorylation (Xu *et al.*, 2012). During the cell cycle, a number of checkpoints ensure that proliferation is regulated, in particular the G1 checkpoint and G1 to S phase transition, which in general prevents unhealthy cells from proceeding further through the cell cycle (Foster *et al.*, 2010). Hypothetically, high levels of either PI(3,4,5)P3 or PI(3,4)P2 present in SHIP-1 modulated cells could indicate that the cell is unhealthy and in order to limit the chance of developing leukaemias these cells are prevented from proliferating. Furthermore, PI3K activity is key to progression through the G1 checkpoint, so a lack of PI(3,4,5)P3 or PI(3,4)P2 may also contribute to cell cycle arrest (Foster *et al.*, 2010). To further this work it would be interesting to use cell cycle analysis to determine the effect of SHIP-1 activation and inhibition upon the cell cycle. Interestingly, expression of complete SHIP-1 protein in Jurkat cells reduced cellular proliferation, notably by affecting the G1 cell cycle regulators Rb and p27^{Kip1} (Horn *et al.*, 2004) and KLF2 (Garcia-Palma *et al.*, 2005) factors implicated in T cell quiescence. The possible reasons for that both pharmacological activation and inhibition SHIP-1 inhibited human T lymphocyte proliferation are summarised in Figure 3.17.

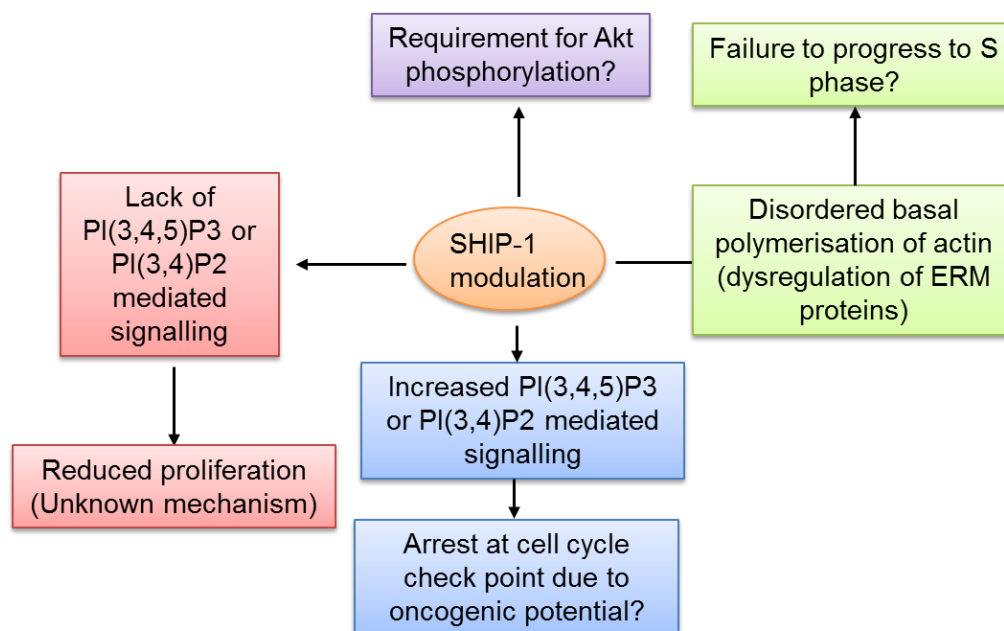


Figure 3.17 Model for SHIP-1 activation and inhibition causing decreased T lymphocyte proliferation

Importantly, although we have indicated that the pharmacological manipulation of SHIP-1 can alter TCR signalling and proliferation, no inappropriate T cell mediated autoimmunity or T cell neoplasm have thus far been demonstrated in either the pharmacological or genetic strategies implemented to manipulate SHIP-1. This includes four independently generated germline SHIP^{-/-} mice strains (Helgason *et al.*, 1998; Liu *et al.*, 1998; Wang *et al.*, 2002b; Karlsson *et al.*, 2003) and conditional T lymphocyte knock out mice, CD4Cre+SHIPflox/flox, or CD8Cre+SHIPflox/flox mice (Tarasenko *et al.*, 2007; Kerr, 2011). It could be argued that such neoplasms are not observed in germline SHIP^{-/-} strains due to their substantially reduced lifespan; however, in the T cell conditional SHIP deletion strains a normal life span is observed. Nor are T cell neoplasms found in primary and secondary hosts reconstituted with SHIP-deficient BM cells (Hazen *et al.*, 2009). Finally, clinical trials with SHIP-1 activator, AQX-1125 have only indicated minor side effects of mild nausea, abdominal pain and dyspepsia (Leaker *et al.*, 2014).

(d) Ca²⁺ signalling

Unlike Akt phosphorylation, TCR-induced Ca²⁺ production was unaffected by SHIP-1 modulation which firstly indicates that Ca²⁺ mobilisation is unlikely to be reliant upon Akt mediated signalling. Consistently to SHIP-1 activation, PI3K deficiency only exhibits a modest effect on Ca²⁺ release into the cytosol in T lymphocytes (Deane *et al.*, 2007). The modest decrease suggests PI3K signalling may not be the only mechanism regulating Ca²⁺ mobilisation. Protein tyrosine kinases SFKs, SYKs and Tec kinase Itk prominently feature in PLC γ activation in T lymphocyte, and although Itk activation and regulation has been associated with both PI3K and SHIP-1 signalling (August *et al.*, 1997; Shan *et al.*, 2000), due to the inability of PI3K deficiency and SHIP-1 modulation to suppress Ca²⁺ release, these roles may be redundant.

SHIP-1 modulates T lymphocyte adhesion by altering the confirmation of the LFA-1 receptor

The adhesion of lymphocytes to components of the extracellular matrix and other cells is critical for cell migration, extravasation and the formation of immunological synapses (Hogg *et al.*, 2011). PI3K-induced signalling is known to be crucial in leukocytes adhesion (Mazerolles *et al.*, 1996), whilst the loss of SHIP-1 expression in neutrophils enhanced adhesion due to up-regulated fibronectin mediated PI(3,4,5)P₃ signalling. SHIP-1^{-/-} neutrophils lose their polarity upon cell adhesion and are extremely adherent which impairs chemotaxis (Mondal *et al.*, 2012). Furthermore, it has been shown that overexpression of full length SHIP-1 had no effect on expression of LFA-1 but increased LFA-1 activity which led to enhance adhesion to ICAM-1 in a manner which was dependent upon the catalytic activity of SHIP-1. However although shRNA SHIP-1 of SHIP-1 reduced expression of CD11a but not CD49d in primary human T lymphocytes it had no effect to adhesion of T lymphocytes to ICAM1 or fibronectin (Harris *et al.*, 2011). Therefore, it was important to address whether pharmacological activation or inhibition of SHIP-1 could alter the ability of human T lymphocytes to adhere.

Surprisingly, both to SHIP-1 activators and inhibitors both potently inhibited the ability of basal and TCR-stimulated T lymphocytes to adhere to ICAM1 and fibronectin. Interestingly, 3AC had a more pronounced effect upon TCR stimulated adhesion, supporting the observation that SHIP-1 is important in TCR induced signalling. These two different adhesion molecules were selected due to their roles in different stages of

T cell extravasation. Fibronectin, a glycoprotein of extracellular matrix and is involved in cell migration, differentiation and early attachment, whereas ICAM-1 is a glycoprotein which is expressed on endothelial and immune cells and is important in stabilising cell contacts. SHIP-1 modulation had no effect upon the expression of respective cognitive integrin receptors of ICAM and fibronectin, LFA-1 (CD11a expression) and $\alpha 4\beta 1$ (CD49d expression) respectively. However, the mechanism behind SHIP-1 modulation inhibiting adhesion to ICAM is due (at least in part) to SHIP-1 altering the LFA-1 avidity. These observations conflict with those previously reported in the literature using genetic targeting. This could be due to differences between human and mouse T lymphocytes, or that genetic targeting of SHIP-1 expression removes both catalytic and non-catalytic functions of SHIP-1, whilst pharmacological manipulation usually leaves non-catalytic functions intact. Due to both the activator and inhibitor of SHIP-1 decreasing the adhesion of T lymphocytes, we propose that, like Akt activation, a balance of phospholipid dependent signalling is required for optimal adhesion and LFA-1 avidity (Figure 3.18).

It is worth mentioning that in physiological conditions the cells would have to adhere under flow and shear stress which could alter requirement/balance of PI3K and SHIP-1 signalling in T lymphocyte adhesion. However as the assay reliably showed enhanced adhesion to TCR stimulation and appeared adequate to determine the effect of SHIP-1 upon T lymphocyte adhesion.

SHIP-1s roles in activated T lymphocyte migration

Chemotaxis is a complex process; PI3K signalling and other pathways often fulfil redundant roles. It has been shown previously that CXCL11-induced migration in human activated T lymphocytes is resistant to pan-PI3K inhibition (Smit et al., 2003; Cronshaw et al., 2006), however we observe that a distinct pan PI3K inhibitor inhibits CXCL11 driven chemotaxis (Discussed in Chapter 5.1). SHIP-1 activation and inhibition abrogated CXCL11-induced directional migration of SEB-activated T lymphocytes in two distinct migration assays. Modulation of SHIP-1 impedes migration in the absence of altering CXCR3 expression or Ca^{2+} signalling, but modulation did significantly reduce CXCL11 mediated Akt phosphorylation.

SHIP-1 activation results are in accordance with recent findings by Lam et al, who demonstrated that overexpression of SHIP in neutrophils impaired migration (Lam et al., 2012), but also with studies indicating SHIP-1 inhibition or genetic targeting with

shRNA mediated knock down inhibits migration of naïve T lymphocytes and neutrophils (Harris *et al.*, 2011; Mondal *et al.*, 2012; Stenton *et al.*, 2013a). Human T lymphocytes migration appears to be highly dependent upon both PI(3,4,5)P3 and PI(3,4)P2 production and signalling. Asymmetric distribution of PI(3,4,5)P3 at the front of the cell is a hallmark of cell polarisation (Wang *et al.*, 2002a; Wang, 2009), and leads to the activation of small GTPases to drive cell migration. PI(3,4)P2 is also important in migration; it selectively recruits TAPP1 which interacts with cytoskeletal protein synotrophin and controls actin polymerisation (Costantini *et al.*, 2009; Li *et al.*, 2013). PI(3,4)P2 also recruits Lammelipodin (Lpd) and promotes dynamic actin branching at the leading edges of migrating cells by interacting with Arp2/WAVE complex (Krause *et al.*, 2004; Law *et al.*, 2013) (Illustrated in Figure 3.18). Thus, both activation and inhibition of SHIP-1 may disrupt T lymphocyte migration (Figure 3.15).

Microvilli on the surface of T lymphocytes collapse in response to chemokine stimulation to allow firm adhesion and flattening of the cell during chemotaxis (Brown *et al.*, 2003). Upon chemokine stimulation, Rac-1 dependent signalling de-phosphorylates ezrin, radixin and moesin (ERM) proteins (Nijhara *et al.*, 2004). When they are phosphorylated ERM proteins link the actin cytoskeleton to the cell membrane phospholipid PI(4,5)P2. De-phosphorylation de-stabilises this attachment and allows microvilli retraction and the formation of a polarised cell (Brown *et al.*, 2003). Thus, ERM de-phosphorylation can be used as indirect marker of microvilli retraction and cellular polarisation. SHIP-1 activation inhibited chemokine (CXCL11 and CXCL12) mediated de-phosphorylation of ERM proteins, this is consistent with shRNA silencing of SHIP-1 protein expression led to a de-phosphorylation of ERM proteins in T lymphocytes (Harris *et al.*, 2011). However, we also observed that pharmacological SHIP-1 inhibition led to abrogated chemokine dependent dephosphorylation of ERM proteins in T lymphocytes. The activity of SHIP-1 therefore seems to be a crucial switch in the phosphorylation state of ERM proteins and thus microvilli retraction within lymphocytes (Figure 3.18).

Microvillus retraction allows LFA-1 (resident on the cell body) to access its ligand, ICAM1 (Lammermann *et al.*, 2014). SHIP-1 activation and inhibition may therefore act to inhibit lymphocyte adhesion to ICAM1 via two mechanisms; reduced LFA-1 avidity, and the inhibition of chemokine mediated ERM de-phosphorylation. In addition the de-phosphorylation of ERM proteins has been shown to be crucial for lymphocyte polarization. Therefore the inability of chemokine mediated ERM de-phosphorylation to occur in the presence of activator and inhibitor, may contribute to the inhibited chemokine mediated migration of lymphocytes.

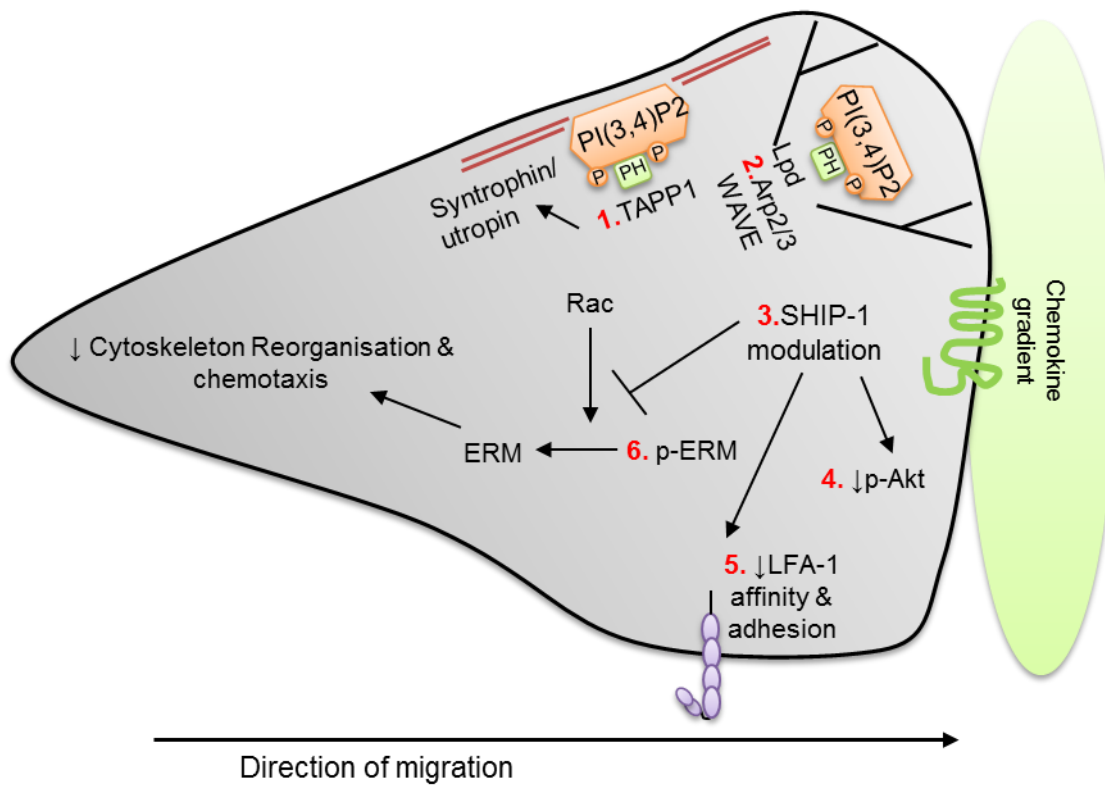


Figure 3.18 Model of the roles of SHIP-1 in T lymphocyte migration and adhesion.

This model is based on the results of the thesis and the findings of (Costantini *et al.*, 2009; Law *et al.*, 2013). **1)** PI(3,4)P2 binding protein TAPP is known to maintain polarized cell morphology via interaction with utrophin and stable F-actin localized at the sides and uropod of migrating cells. **2)** Lammellipodin (Lpd), another PI(3,4)P2 binding protein, promotes dynamic actin branching at leading edges (lammellipodia) of migrating cells via its interaction with Arp2/3 WAVE complex. **3)** SHIP-1 was pharmacologically manipulated by SHIP-1 activator, AQX1 or SHIP-1 inhibitor 3AC. **4)** SHIP-1 modulation impaired CXCL11-induced Akt phosphorylation and **5)** reduced the avidity of integrin LFA-1 resulting in reduced basal adhesion. **6)** SHIP-1 modulation prevented pERM dephosphorylation to CXCL11, which is likely to alter cytoskeletal reorganisation. No effect upon CXCR3 receptor expression or Ca^{2+} mobilization was observed. SHIP-1 appears to be critical in T lymphocyte migration.

Perspectives:

SHIP-1 has a crucial role in the regulation of ligand mediated signalling, proliferation, adhesion, cytoskeletal protein re-arrangement and migration of primary human T lymphocytes. Consequently, pharmacological modulation of SHIP-1 offers an exciting new mechanism for the targeted modulation of PI3K signalling, with potential therapeutic opportunities in T lymphocyte driven pathologies such as autoimmune and inflammatory disease.

Interestingly, over the duration of this project intense research has continued to produce evidence that the PI3K, particularly δ and γ isoforms, have important non-redundant role in multiple cells of the immune system and could be useful in the treatment immune pathologies [We have reviewed this area here (Ball *et al.*, 2014)]. The growing appreciation of the isoform crystal structures of PI3K, which help define the structure-activity rules for obtaining selectivity, continuously enhance our ability to design and develop of PI3K inhibitors with greater isoform selectivity and fewer off-target effects (Sutherlin *et al.*, 2012; Zhao *et al.*, 2014; Somoza *et al.*, 2015; Yang *et al.*, 2015).

However, we believe that even with improved PI3K inhibitors, there are potential benefits to targeting SHIP-1. Firstly, there is a large amount of functional redundancy and plasticity between PI3K isoforms, thus isoform targeted therapies might not warrant successful treatments in disease setting. Moreover, PI3K dysregulation is often caused by over-active mutations in PI3K (Pazarentzos *et al.*, 2015; Young *et al.*, 2015), these could alter the efficacy and affinity of PI3K inhibitors. Furthermore, the ubiquitous nature of PI3K signalling implies that any successful PI3K inhibitors could be complicated by off-target effects and worryingly, it has recently been reported that resistance mechanisms can be developed to combat PI3K-targeted therapy (Ilic *et al.*, 2011); although selective pressure driving resistance could vary between cancer and inflammatory setting. SHIP-1 exhibits a restricted expression profile limiting non-selective effects and modulation of SHIP-1 appears to have specific effects on the immune system. SHIP-1 could be a superior target, and at the very least it will be beneficial to have multiple mechanisms to pharmacologically target PI3K signalling.

CHAPTER 4: Examining the functional consequences of ROS manipulation on T lymphocyte biology

4.1 Rationale

Reactive oxygen species (ROS) are known to influence the outcome of T-cell responses. Depending on concentration, exposure time and microenvironment, the effects of ROS on T lymphocytes can be very distinct. T lymphocytes produce low levels of hydrogen peroxide upon TCR and chemokine stimulation (Hara-Chikuma *et al.*, 2012) and are known to express NADPH oxidase enzymes NOX2 (Jackson *et al.*, 2004) and DUOX 1 (Kwon *et al.*, 2010). High levels of ROS have a putative role in several T lymphocyte induced pathologies including autoimmune diseases, leukaemias, carcinoma, cardiovascular diseases and neurological disorders.

Historically, ROS were viewed as an unwanted by-product of aerobic respiration, which caused cellular damage via oxidation. However there has been a growing body of evidence to suggest that ROS are actually required for a vast number of physiological processes, including TCR activation and signalling. Antioxidants inhibit T lymphocyte proliferation and IL-2 production in humans and mice (Dornand *et al.*, 1989; Tatla *et al.*, 1999; Sena *et al.*, 2013). A very recent study has indicated that ROS is not required in TCR-induced proliferation, activation and cytokine secretion of primary human T lymphocytes (Belikov *et al.*, 2014); before this study the roles of ROS and antioxidants on TCR signalling and responses had not been extensively investigated in primary human T lymphocytes.

H₂O₂ has been observed to be an early danger cue required for neutrophil recruitment to wounds in zebrafish (Niethammer *et al.*, 2009). Furthermore, a recent finding suggested that H₂O₂ is required for successful mouse T lymphocyte migration towards a chemoattractant (Hara-Chikuma *et al.*, 2012). To date, little is known about whether hydrogen peroxide is required for the migration of human T lymphocytes.

In order to examine the effect of ROS, primary human T lymphocytes were isolated from healthy human peripheral blood. Two types of primary T lymphocytes were used 1) freshly isolated “naïve” and 2) SEB-activated T lymphocytes. Limitations of working with naïve T lymphocytes are they have to be used on the day of isolation following a lengthy isolation process; it is also difficult to obtain large numbers of naïve T lymphocytes. Finally, naïve T lymphocytes function to survey the blood and lymphoid systems for antigen and are less likely to be at the site of infection exposed to high levels of ROS. Therefore, peripheral blood mononuclear cells were treated with *staphylococcal enterotoxin B (SEB)*, a super-antigen produced by the gram-positive bacteria *Staphylococcus aureus*, for 36 hours. SEB is a strong polyclonal activator of T lymphocytes and after activation the cells are clonally expanded in the presence of IL-

2. SEB-activated T lymphocytes were used to model effector T lymphocyte migrating in infected tissues which is likely to be exposed to higher levels of ROS.

4.1.1 Aims

This chapter aims to determine the consequences of ROS manipulation on human T lymphocyte biology.

Several pharmacological manipulators of ROS will be used:

- to establish the functional roles of ROS in human T lymphocyte viability and TCR activation including investigating downstream Ca^{2+} elevation, proliferation and adhesion.
- to determine whether H_2O_2 acts as a chemoattractant in T lymphocytes.
- to assess the functional importance of ROS as a regulator of basal and chemotactic migration.
- to establish whether H_2O_2 alters CXCL11-induced Ca^{2+} elevation or actin regulation.

4.2 ROS manipulators alter intracellular ROS accumulation in SEB-activated T lymphocytes

During Chapter 4 and Chapter 5, pharmacological manipulators of ROS will be described and their effects on T lymphocyte functions assessed. Therefore it was prudent to determine the compounds efficacy at manipulating intracellular ROS levels. Figure 4.1 illustrates the regulation of ROS by the pharmacological manipulators used. A 2',7'-dichlorodihydrofluorescein diacetate ($H_2DCF\text{-}DA$ – “DCF”) based fluorescence assay was used to detect intracellular ROS in SEB-activated T lymphocytes.

Cells were loaded with $1\mu M$ H_2DCFDA , which is cleaved by intracellular esterases at the two ester bonds to produce H_2DCF , a polar and cell membrane-impermeable product which is retained within the cell. Subsequent oxidation converts H_2DCF to the highly fluorescent DCF. Cells were plated into a black 96 well plate and incubated with increasing concentrations H_2O_2 for 30 minutes. H_2O_2 was added to verify the activity of the H_2DCF dye, which should detect an increased accumulation of ROS due to the diffusion of H_2O_2 into the cell. Positive control H_2O_2 increased intracellular ROS levels as compared to untreated controls (Figure 4.2 A).

Next, the cell permeable superoxide dismutase (SOD) mimetic, [5,10,15,20-tetrakis(4-carboxyphenyl)-21*H*,23*H*-porphine]manganese(III) chloride ($C_{48}H_{26}ClMnN_4O_8$, MnTBAP chloride) was validated. This compounds has been previously used here (Liu *et al.*, 2013; Schrammel *et al.*, 2013; Suresh *et al.*, 2013). SOD is an enzyme that catalyzes the dismutation of superoxide radicals into H_2O_2 and O_2 , enhancing intracellular H_2O_2 levels. MnTBAP chloride has an EC_{50} of $40\mu M$ reported by the manufacturer (Abcam). It is important to note MnTBAP chloride is also a peroxynitrite scavenger. Cells were treated for 30 minutes with MnTBAP chloride and a significant albeit modest intracellular ROS elevation was observed (Figure 4.2 B). However, DCF is unable to specifically detect H_2O_2 , the major outcome of MnTBAP chloride on overall ROS level is anti-oxidant as it reduces the level of superoxide to produce H_2O_2 .

Two different compounds were utilised to dampen the redox environment: (1) diphenyleneiodonium (DPI), a NOX inhibitor, was used to block superoxide production. The K_i for time dependent inhibition of NOX in neutrophils is $5.6\mu M$ (O'Donnell *et al.*, 1993). However, DPI also inhibits other flavoenzymes (2) Catalase, an enzyme that rapidly catalyses the decomposition of H_2O_2 to water and oxygen, was used to reduce levels of H_2O_2 . Catalase was used at recommended active concentration reported by the manufacturer (Sigma Aldrich). Cells were treated with DPI for 30 minutes and no effect upon basal ROS production was observed (Figure 4.2 C).

Flow cytometry was used to validate catalase-dependent reductions in intracellular ROS, as a previous control experiment indicated that catalase has auto-fluorescence in the FITC range. Using flow cytometry the live cell population was gated, avoiding the auto-fluorescence of catalase, which has a much smaller forward and side scatter than live cells. Cells were treated with 1mg/ml catalase and DCF fluorescence intensity measured every 5 minutes for 30 minutes. Catalase significantly reduced basal ROS accumulation over time (Figure 4.2 D). Catalase is highly efficient and cannot be saturated by H_2O_2 at any concentration (Lledias *et al.*, 1998). Cells were pre-treated with 1mg/ml catalase for 30 minutes then treated with 10mM H_2O_2 for 30 minutes. Catalase pre-treatment completely attenuated the increase in DCF fluorescence observed with H_2O_2 alone (Figure 4.2 E). These findings confirm that these are suitable agents to manipulate ROS in T lymphocytes.

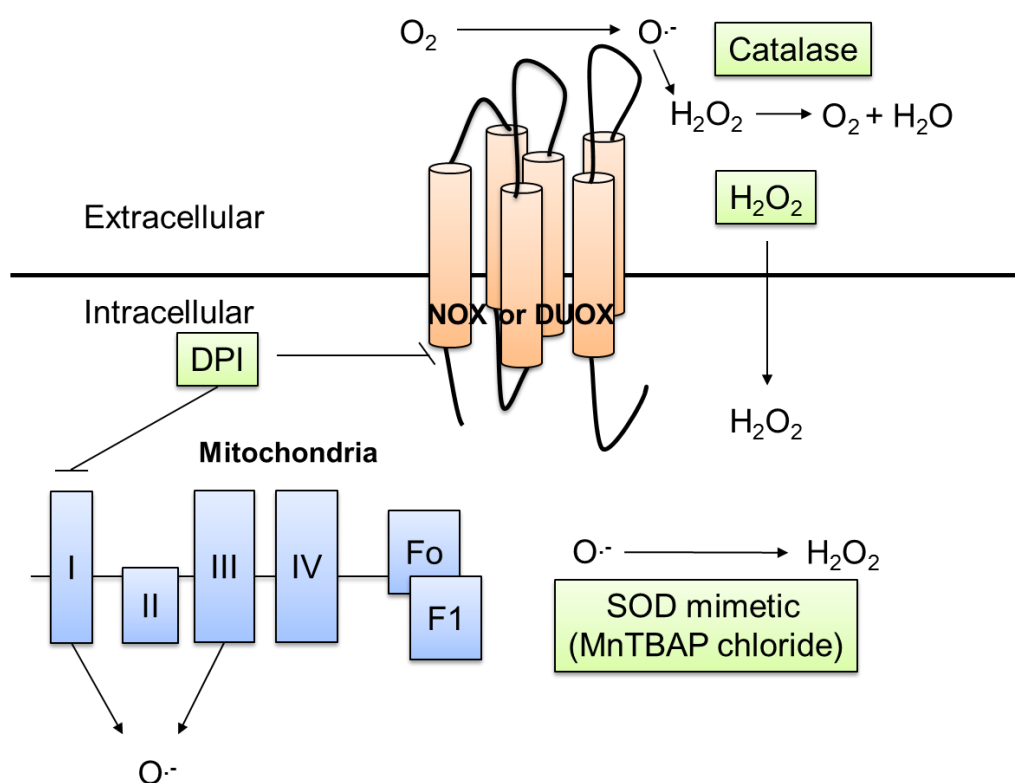


Figure 4.1 Regulation of ROS by pharmacological manipulators. Extracellular treatment of H_2O_2 will increase H_2O_2 concentration both outside and inside the cell as it enters through aquaporins or diffusion. Superoxide dismutase (SOD) is an enzyme that catalyzes the dismutation of superoxide radicals into H_2O_2 and O_2 . Here SOD mimetic MnTBAP chloride is used to enhance the intracellular levels of H_2O_2 . DPI is a flavoenzyme inhibitor which inhibits ROS production from NOX/DUOX enzymes (that are expressed on cellular membranes) and the electron transport chain in the mitochondria. Catalase is an enzyme that rapidly catalyses the decomposition of H_2O_2 to water and oxygen which reduce levels of extracellular H_2O_2 . The pharmacological mediators are highlight by green boxes.

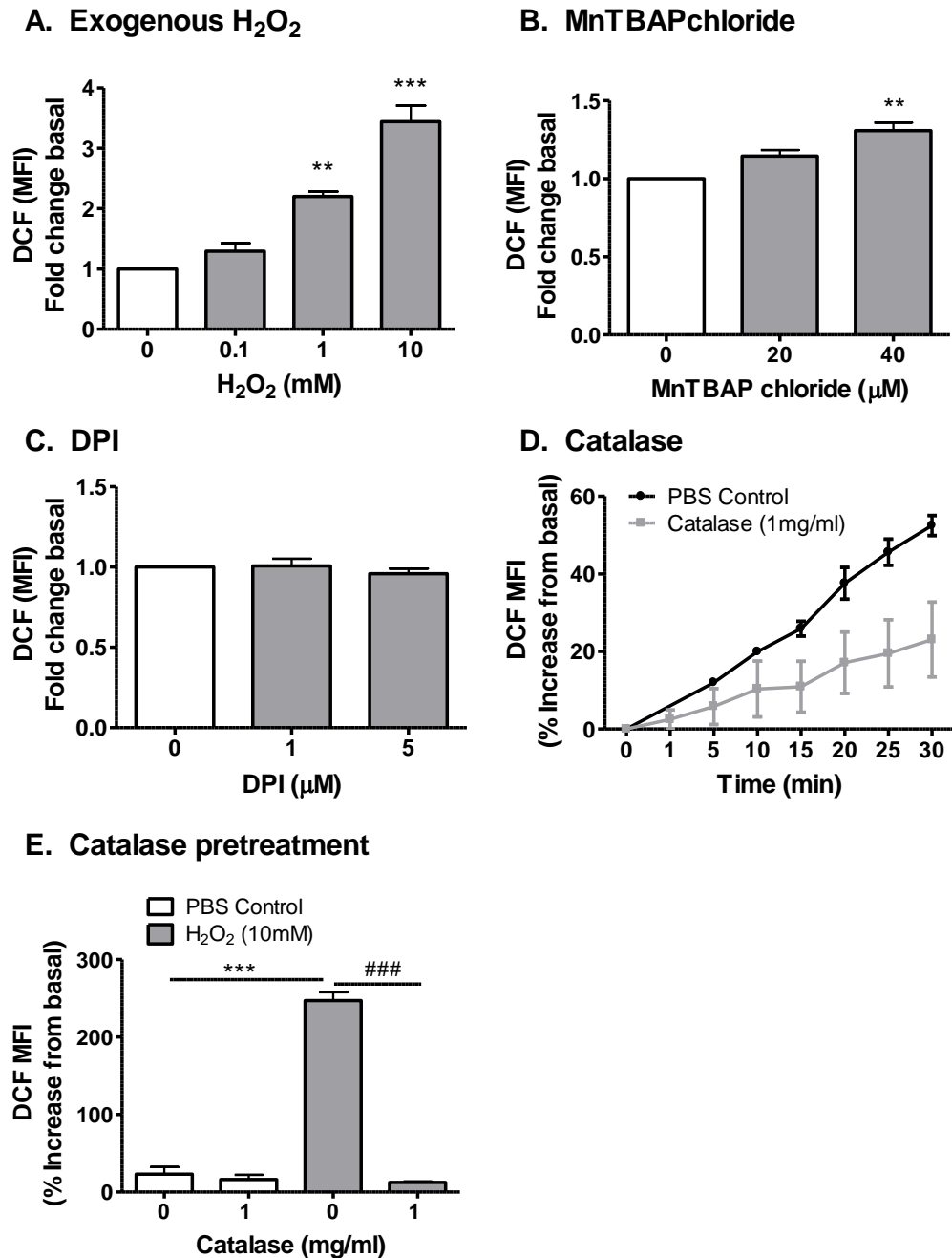


Figure 4.2 Intracellular ROS is increased by H_2O_2 / MnTBAP chloride and reduced by catalase in SEB-activated T lymphocytes. SEB-activated T lymphocytes (8-12 days post isolation) were loaded with 1μ M DCFDA for 30 minutes. Cells were washed twice in HBSS, resuspended at 1 million cells per ml and treated with stated concentrations of either (A) H_2O_2 , (B) MnTBAP chloride, (C) DPI or Catalase (D & E). For (A, B & C) ROS generation was measured after 30 minutes using a plate reader and normalised to fold change from basal. For (D & E), ROS generation was measured using flow cytometry. (D) Cells were either treated with PBS control or catalase at time point 0. Fluorescence intensity was then read every 5 minutes for 30 minutes and plotted as percent increase from basal. (E) Cells were pre-treated either with PBS control or catalase for 30 minutes and basal fluorescence reading taken. Then cells were treated with 10mM H_2O_2 for 5 minutes before fluorescence was measured. Data are the mean \pm SEM of three independent experiments. Statistical significance was determined by a one-way ANOVA with Dunnett's post-test where ** $p < 0.01$ or *** $p < 0.001$ as compared to control or ### $p < 0.001$ as compared to H_2O_2 alone

4.3 Effect of ROS manipulation on SEB-activated T lymphocyte viability

As ROS are known cytotoxic agents able to induce cellular death, it was imperative to observe whether the concentrations of ROS manipulators used in the following experiments alter cellular viability. In addition, the roles of ROS in human T lymphocyte survival were also examined. Two different cell viability assays were utilised; the (1) MTT assay and (2) Annexin V/ propidium iodide staining.

4.3.1 ROS manipulation upon cell viability assessed by the MTT assay

Firstly, cells were treated with increasing concentration of the ROS manipulators for either 3 or 24 hours, followed by determination of cellular viability by MTT assay (described in section 3.3.1). Cell viability was determined after 3 hours, as this is the maximum exposure time to ROS manipulators in all experiments (with the exception of Figure 4.6-9), and after 24 hours. Unfortunately, the effect of MnTBAP chloride upon cell viability could not be assessed in the MTT assay due to the compounds brown colour affecting the validity of the colorimetric assay by enhancing absorbance measurements.

After a 3 hour treatment, cell viability was unaffected by H_2O_2 up to concentrations of $100\mu\text{M}$. Cell survival decreased to $38.2 \pm 1\%$ following stimulation with 1mM H_2O_2 (Figure 4.3 A). However after a 24 hour treatment with H_2O_2 , enzymatic activity was significantly reduced at concentrations above $10\mu\text{M}$ H_2O_2 (Figure 4.3 B). This implies that the survival of T lymphocytes treated with H_2O_2 depends on both the concentration and the exposure time.

Cells were treated with increasing concentrations of catalase for either 3 or 24 hours to observe the effect of dampening intracellular ROS on cell viability. After a 3 hour treatment, catalase had no effect upon cellular viability (Figure 4.3 C). Interestingly, 24 hour treatment of catalase caused a small but concentration dependent reduction in cell viability (Figure 4.3 D). To identify whether responses are catalase-dependent the catalase was heat inactivated and denatured by boiling it for 10 minutes. 24 hours treatment with heat-inactivated catalase had no effect upon cell viability (Figure 4.3.E).

Finally, cells were treated with increasing concentrations of DPI, for either 3 or 24 hours, to determine the effect of inhibiting ROS production on cellular viability. 3 hour treatment with DPI had no effect upon cellular viability (Figure 4.3 F), whereas 24 hour treatment of DPI resulted a small but significant reduction in cell viability at both

concentrations examined (Figure 4.3 G). This implies that ROS production and presence is required for optimal cell survival.

4.3.2 ROS manipulators had no effect upon PI/AV staining

As the MTT assay is redox based, results could be skewed if ROS treatment were still present. Therefore, alternative assays were used to assess viability, namely annexin V and propidium iodide staining with is described in section 3.3.2. Cells were treated with increasing concentrations of ROS manipulators; H_2O_2 , MnTBAP chloride, catalase and DPI for 3 hours. Annexin V staining was unaffected at all concentrations of H_2O_2 below 10mM and MnTBAP chloride, catalase and DPI had no effect upon annexin V staining (Figure 4.4 & Figure 4.5).

Consistently with the annexin V staining, PI staining was unaffected at all concentrations of H_2O_2 below 10mM and MnTBAP chloride, catalase and DPI had no effect upon PI staining at all examined concentrations (Figure 4.4 & Figure 4.5). This confirmed that observations obtained with the MTT assay and suggested that functional responses observed with ROS manipulation were not due to them altering cellular viability.

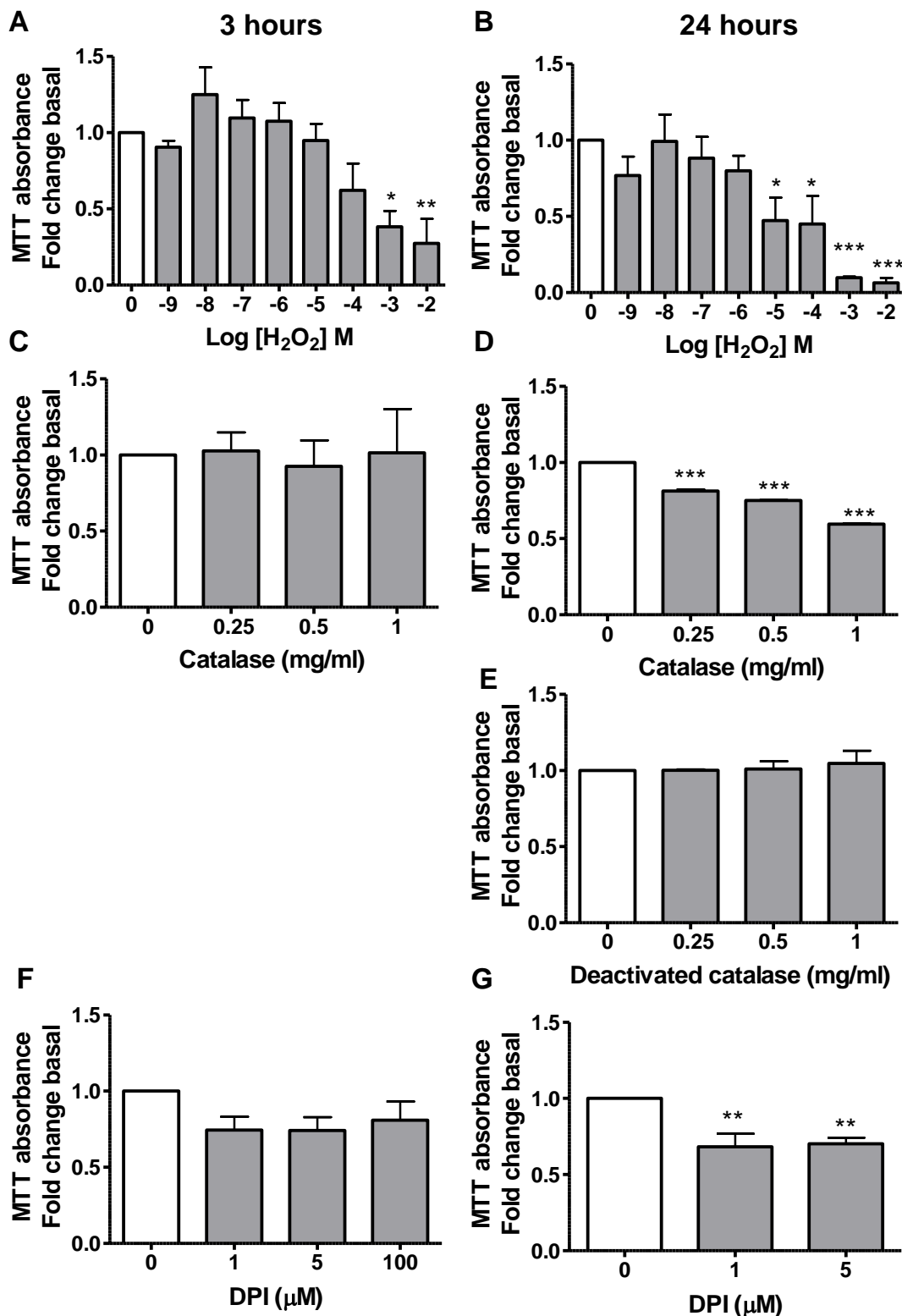


Figure 4.3 Effect of ROS manipulators on SEB-activated T lymphocyte viability using the MTT assay. 1 million SEB-activated T lymphocytes (8-12 days post isolation) were treated with increasing concentrations of (A, B) H₂O₂, (C,D) catalase, (E) deactivated catalase or (F, G) DPI for either 3 hours or 24 hours, respectively. Cells viability was assessed using the MTT assay. Data are mean ± SEM from three independent donors. Statistical significance was determined using a one-way ANOVA with Dunnett's post-test where *p<0.05, **p<0.01 or ***p<0.001 compared to control.

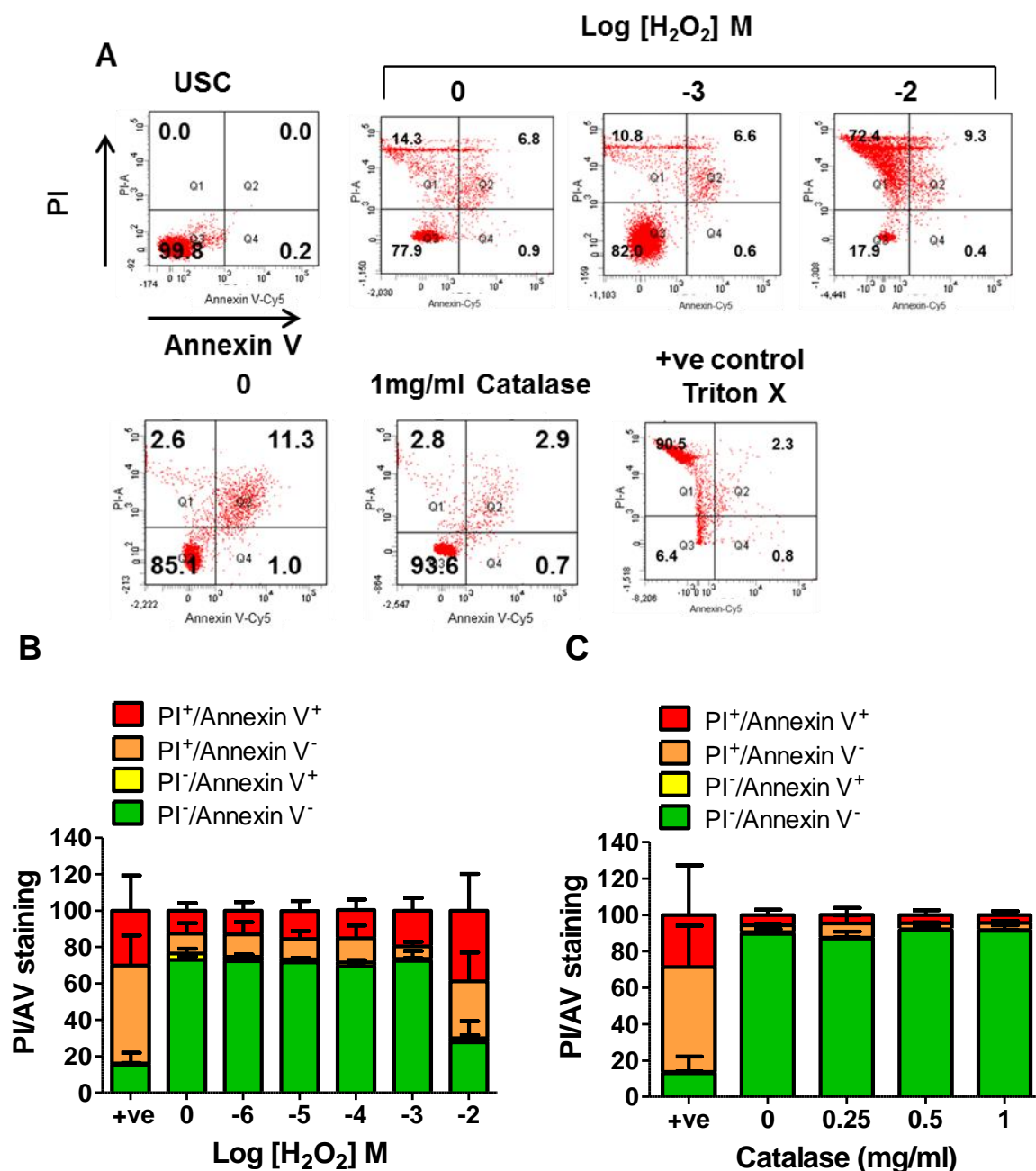


Figure 4.4 Measurements of Annexin V and PI staining indicates that high concentrations of H₂O₂ are cytotoxic, but catalase is non-cytotoxic to SEB-activated T lymphocytes. 1 million SEB-activated T lymphocytes (8-12 days post isolation) were treated with increasing concentrations of (B) H₂O₂ or (C) catalase plus 1% Triton X as a positive control for 3 hours. Cells were stained with Annexin V and PI, and fluorescence quantified by flow cytometry. (A) Representative FACS plots are given, including an unstained control (USC) and positive control 1% Triton X (+ve). The mean± SEM percentage of cells which were PI⁻/Annexin V⁻ are shown in green, PI⁻/Annexin V⁺ (yellow), PI⁺/Annexin V⁻ (orange), and PI⁺/Annexin V⁺ (Red) for cells treated with increasing concentrations of (B) H₂O₂ (n=4) or (C) catalase (n=3).

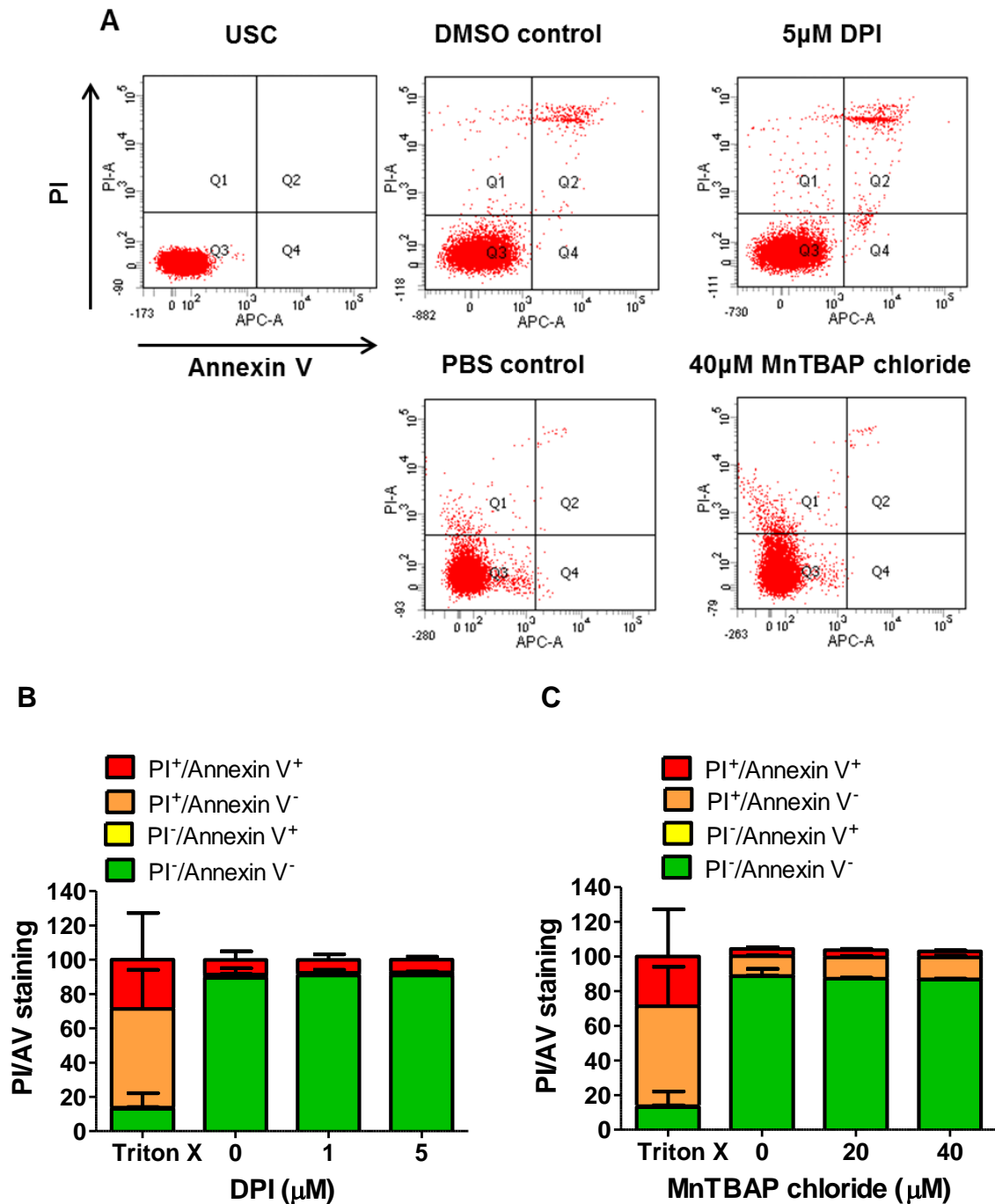


Figure 4.5 Measurement of Annexin V and PI staining indicates that DPI and MnTAB chloride are non-cytotoxic to SEB-activated T lymphocytes. 1 million SEB-activated T lymphocytes (8-12 days post isolation) were treated with stated concentrations of DPI or MnTBAP chloride for 3 hours. Cells were stained with Annexin V and PI, and fluorescence quantified by flow cytometry. (A) Representative dual PI/Annexin V stained plots are given, including an unstained control (USC). The mean \pm SEM percentage of cells which were PI⁻/Annexin V⁻ are shown in green, PI⁻/Annexin V⁺ (yellow), PI⁺/Annexin V⁻ (orange), and PI⁺/Annexin V⁺ (Red) for cells treated for 3 independent experiments.

4.4 Effect of ROS manipulation upon TCR-induced responses

Ligation of the TCR receptor induces signalling required for the activation, survival and proliferation of T lymphocytes. TCR ligation has been shown to induce intracellular ROS (Jackson *et al.*, 2004) and accumulating evidence suggested ROS are actually required for TCR activation and signalling (Kwon *et al.*, 2010; Sena *et al.*, 2013). Therefore the functional roles of ROS and antioxidants downstream of CD3/CD28 signalling in human T lymphocytes were investigated.

4.4.1 H₂O₂ has no effect upon TCR-induced Ca²⁺ elevation

Intracellular Ca²⁺ was measured to assess whether H₂O₂ affects TCR signalling potential. In Chapter 3 Figure 3.5A, UCHT-1 was observed to induce a sustained, concentration dependent increase in intracellular Ca²⁺. To test whether H₂O₂ could affect this UCHT-1 response, SEB-activated T lymphocytes were pre-treated with H₂O₂ for 30 minutes. H₂O₂ had no effect upon UCHT-1-induced Ca²⁺ elevation at any concentration studied (Figure 4.6). This implies that H₂O₂ does not affect TCR signalling through Ca²⁺ signalling.

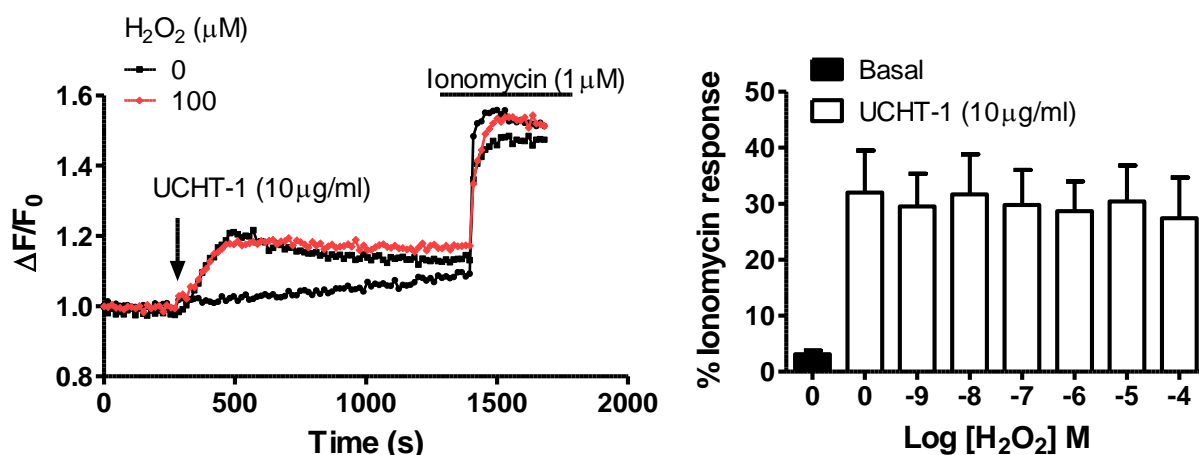


Figure 4.6 H_2O_2 had no effect upon TCR agonist (UCHT-1)-induced intracellular Ca^{2+} release in SEB-activated T lymphocytes. SEB-activated T lymphocytes (8-12 days post isolation) were washed into PBS and loaded for 45 minutes with $1\mu\text{M}$ Fluo-4. Cells were washed 3 times and re-suspended at 1 million cells per ml in Ca^{2+} free-HBSS and extracellular Ca^{2+} adjusted to 1mM . Cells were plated in a black 96 well plate and either allowed to rest or treated with increasing concentrations of H_2O_2 for 30 minutes. $10\mu\text{g/ml}$ UCHT-1 was added after a baseline was established and $1\mu\text{M}$ ionomycin was used as a loading control. Left panel is a kinetic trace indicating change in fluorescence from basal ($\Delta F/F_0$) from one representative donor. The right panel is the peak change in fluorescence generated by UCHT-1 as a percentage of the peak change generated by ionomycin and is mean \pm SEM from averaged duplicates of three independent donors. Statistical significance was tested using a one-way ANOVA with Dunnett's post-test relative to control.

4.4.2 TCR-induced proliferation of naïve T lymphocytes is impaired by exogenous H₂O₂ but not by MnTBAP chloride

Next, the effect of ROS manipulation was assessed in the clonal expansion of human T lymphocytes, to examine another TCR/CD3 mediated event. Naïve T lymphocytes were treated with increasing concentrations of H₂O₂ for 30 minutes before stimulation with anti-CD3/CD28 Ab coated Dynabeads and IL-2. CFSE intensity was determined four days post isolation with plots from control conditions showing four distinct peaks, indicating four generations of cell division (Figure 4.7 A). H₂O₂ (10µM) increased the percentage of cells retained in generation 1 resulting in fewer cells progressing to generation 4 (Figure 4.7 B). H₂O₂ (10µM) also caused a significant increase in the CFSE mean fluorescence intensity, indicating cells have been unable to divide and thus retained a higher level of CFSE (Figure 4.7 C). Together, this suggests that at concentrations equal and above 10µM, H₂O₂ inhibits the ability of human CD4 lymphocytes to proliferate and progress through cell divisions upon TCR/CD3 stimulation. As H₂O₂ (10µM) significantly decreases cell viability at 24 hours, this could be due in part to cytotoxicity (Figure 4.3).

Next, cells were treated with 20µM and 40µM MnTBAP chloride for 30 minutes prior to TCR stimulation to observe whether raising intracellular H₂O₂ would have the same effect as exposure to exogenous H₂O₂. CFSE intensity was determined five days post isolation for this experiment and five distinct peaks can be observed in the FACs plots from control conditions (Figure 4.8 A). MnTBAP chloride had no effect on the percentage of cells within each division, with the majority of cells progressing to generation 5 (Figure 4.8 B), and no effect upon CFSE fluorescence, with all conditions having a low mean intensity (Figure 4.8 C). Unlike H₂O₂, MnTBAP chloride had no effect upon the ability of naïve CD4 lymphocytes to proliferate, however MnTBAP chloride did only induce a marginal increase in intracellular ROS as compared to exogenous H₂O₂ (Figure 4.2) and had no effect upon cellular viability (Figure 4.2).

4.4.3 Catalase and NADPH oxidase inhibitor (DPI) inhibit TCR-induced proliferation of naïve T lymphocytes

Naïve T lymphocytes treated with increasing concentrations of catalase for 30 minutes before stimulation with anti-CD3/CD28 coated Dynabeads and IL-2 to examine whether dampening intracellular ROS impacts T lymphocyte proliferation. CFSE intensity was determined five days post isolation with plots from control conditions showing five distinct peaks indicating five generations of cell division (Figure 4.9 A). Catalase increased the percentage of cells retained in generation 1; whereas fewer cells progressed to generation 4 (Figure 4.9 B). Catalase also caused a significant increase in the CFSE mean fluorescence intensity indicating cells have retained a higher level of CFSE by being unable to divide (Figure 4.9 C). This indicates that decreasing intracellular ROS inhibited the ability of human CD4 lymphocytes to proliferate and progress through cell divisions upon TCR/CD3 stimulation.

Finally, the effect of the NOX inhibitor DPI upon naïve T lymphocyte proliferation was observed. DPI potently inhibited the ability of human CD4 lymphocytes to proliferate, with the CFSE intensity FACs plots only showing two peaks as compared to the five distinct peaks in the control condition (Figure 4.10 A). DPI increased the percentage of cells retained in generation 1; whereas only a few cells were seen in generation 2 and 3 and no cells progressed to generation 4 (Figure 4.10 B). DPI also significantly enhanced the CFSE mean fluorescence intensity indicating that cells have retained a higher level of CFSE by being unable to divide (Figure 4.9 C). This indicates that decreasing ROS production inhibits the ability of human CD4 lymphocytes to proliferate and progress through cell divisions upon TCR/CD3 stimulation.

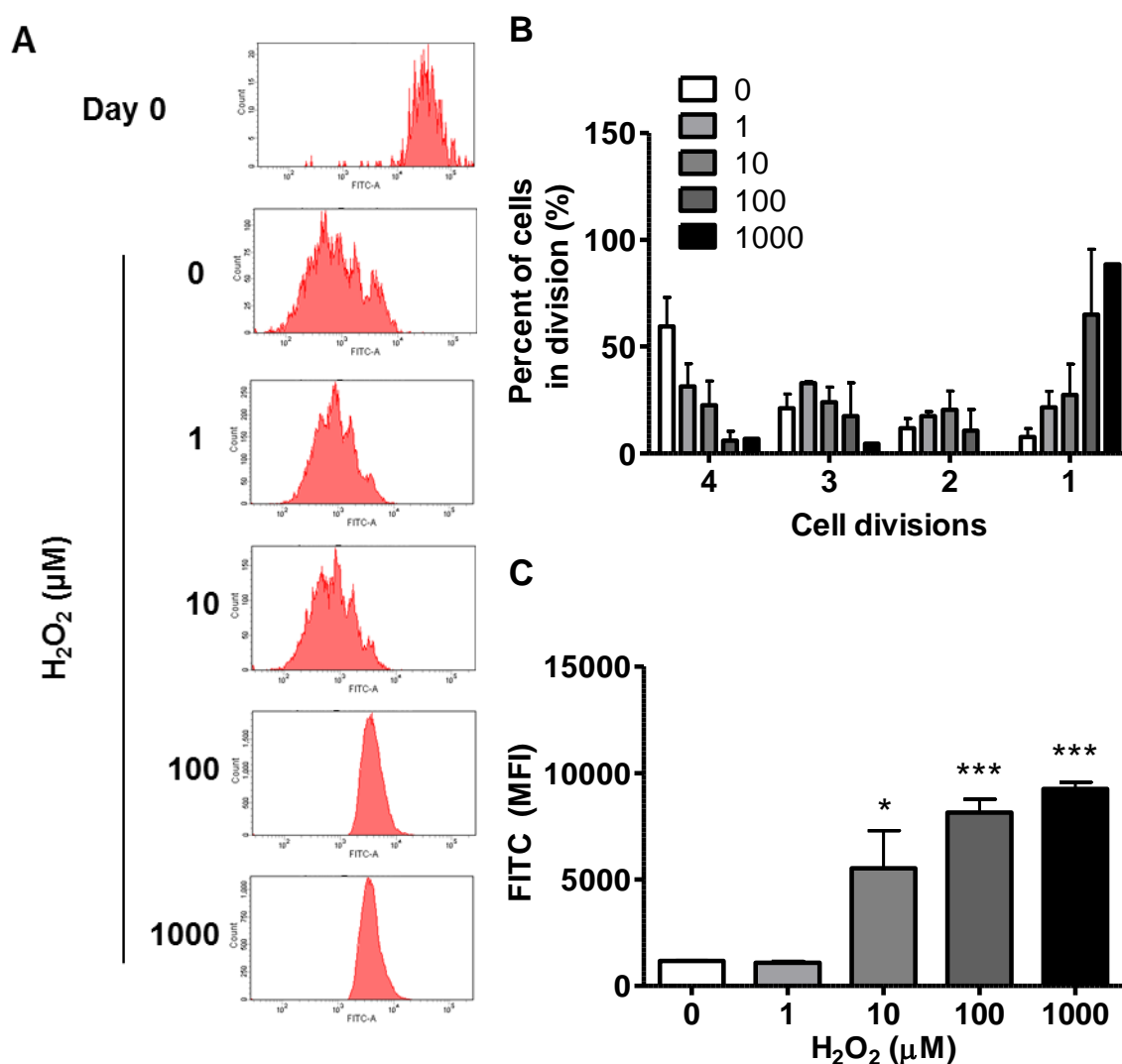


Figure 4.7 H₂O₂ impairs TCR-induced proliferation of Naive T lymphocytes. Freshly isolated naïve CD4⁺ T lymphocytes were labelled with CFSE. 1 million cells were analysed on the same day (Day 0) or pre-treated with vehicle (media) or increasing concentrations of H₂O₂ for 30 minutes, followed by addition of anti-CD3/CD23 antibody coated beads to cells at a ratio of 3:1. IL-2 was added at a final concentration of 36 Units/ml and cells cultured for 4 days before analysis of CFSE fluorescence. Data are (A) representative FACs plots from one donor, (B) mean \pm SEM percentage of cells in each cell division and (C) CFSE mean \pm SEM fluorescence intensity from three independent donors. Statistical significance was determined by one way-ANOVA with Dunnett's post-test where * p <0.05 and *** p <0.001 compared to control.

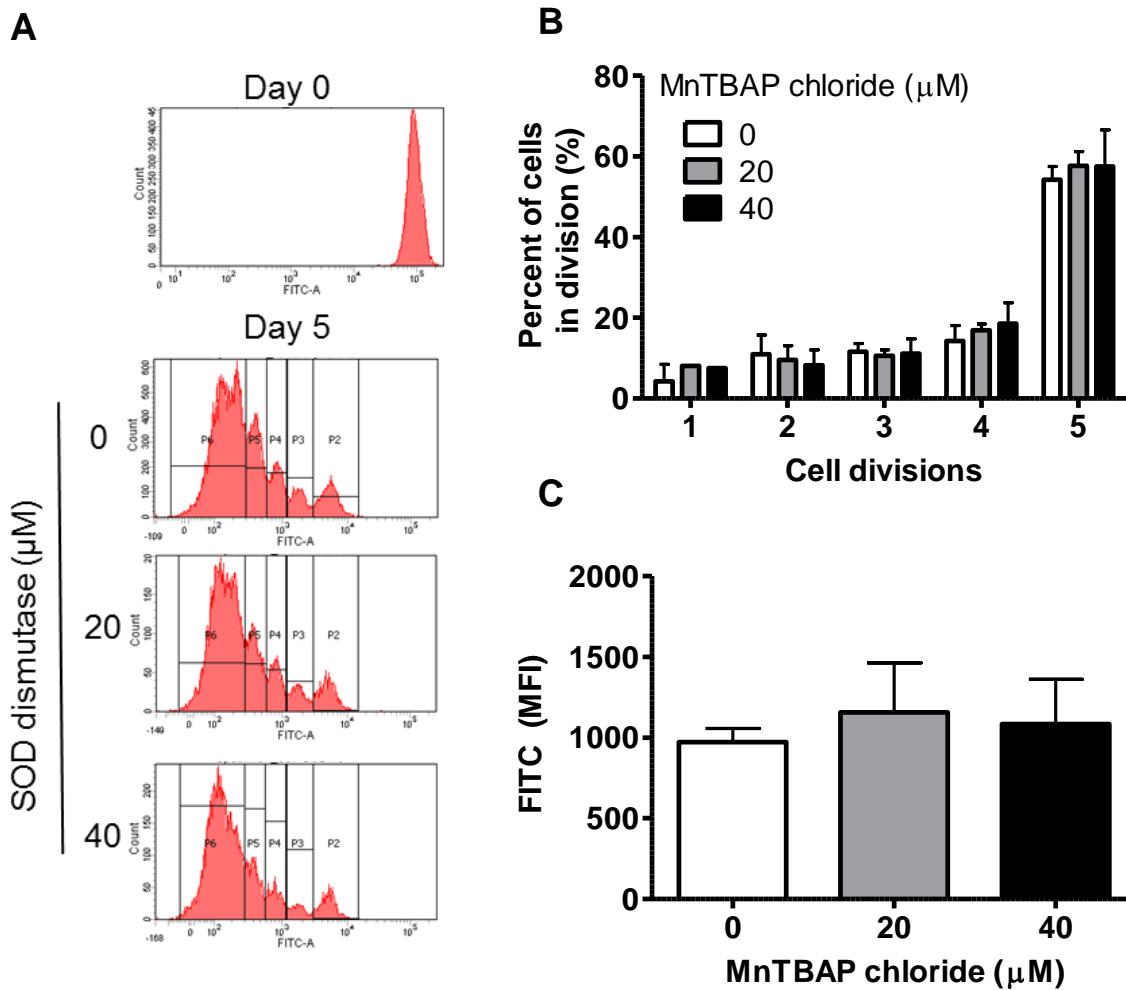


Figure 4.8 MnTBAP chloride had no effect upon TCR-induced proliferation of naive T lymphocytes. Freshly isolated naive CD4⁺ T lymphocytes were labelled with CFSE. 1 million cells were analysed on the same day (Day 0) or pre-treated with vehicle (media) or increasing concentrations of MnTBAP chloride for 30 minutes, followed by addition of anti-CD3/CD23 antibody coated beads to cells at a ratio of 3:1. IL-2 was added at a final concentration of 36 Units/ml and cells cultured for 5 days before analysis of CFSE fluorescence. Data are (A) representative FACS plots from one donor, (B) mean \pm SEM percentage of cells in each cell division and (C) CFSE mean \pm SEM fluorescence intensity from three independent donors. Statistical significance was determined by one way-ANOVA with Dunnett's post-test relative to control.

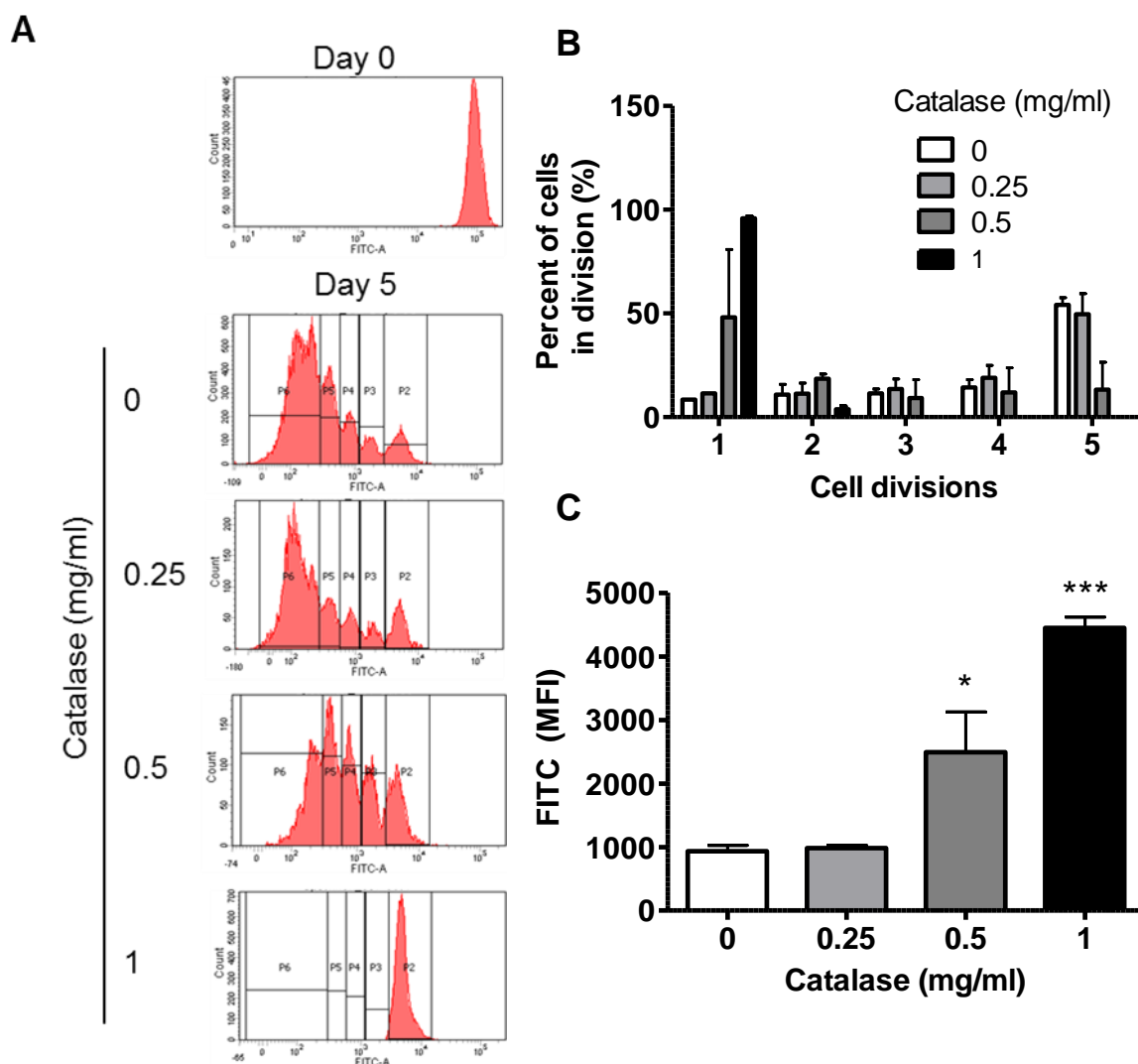


Figure 4.9 Catalase significantly impaired naïve T lymphocyte proliferation. Freshly isolated naïve CD4⁺ T lymphocytes were labelled with CFSE. 1 million cells were analysed on the same day (Day 0) or pre-treated with vehicle (media) or increasing concentrations of Catalase for 30 minutes, followed by addition of anti-CD3/CD23 antibody coated beads to cells at a ratio of 3:1. IL-2 was added at a final concentration of 36 Units/ml and cells cultured for 5 days before analysis of CFSE fluorescence. Data are (A) representative FACS plots from one donor, (B) mean \pm SEM percentage of cells in each cell division and (C) CFSE mean \pm SEM fluorescence intensity from three independent donors. Statistical significance was determined by one way-ANOVA with Dunnett's post-test where * $p < 0.05$ and *** $p < 0.001$ compared to control.

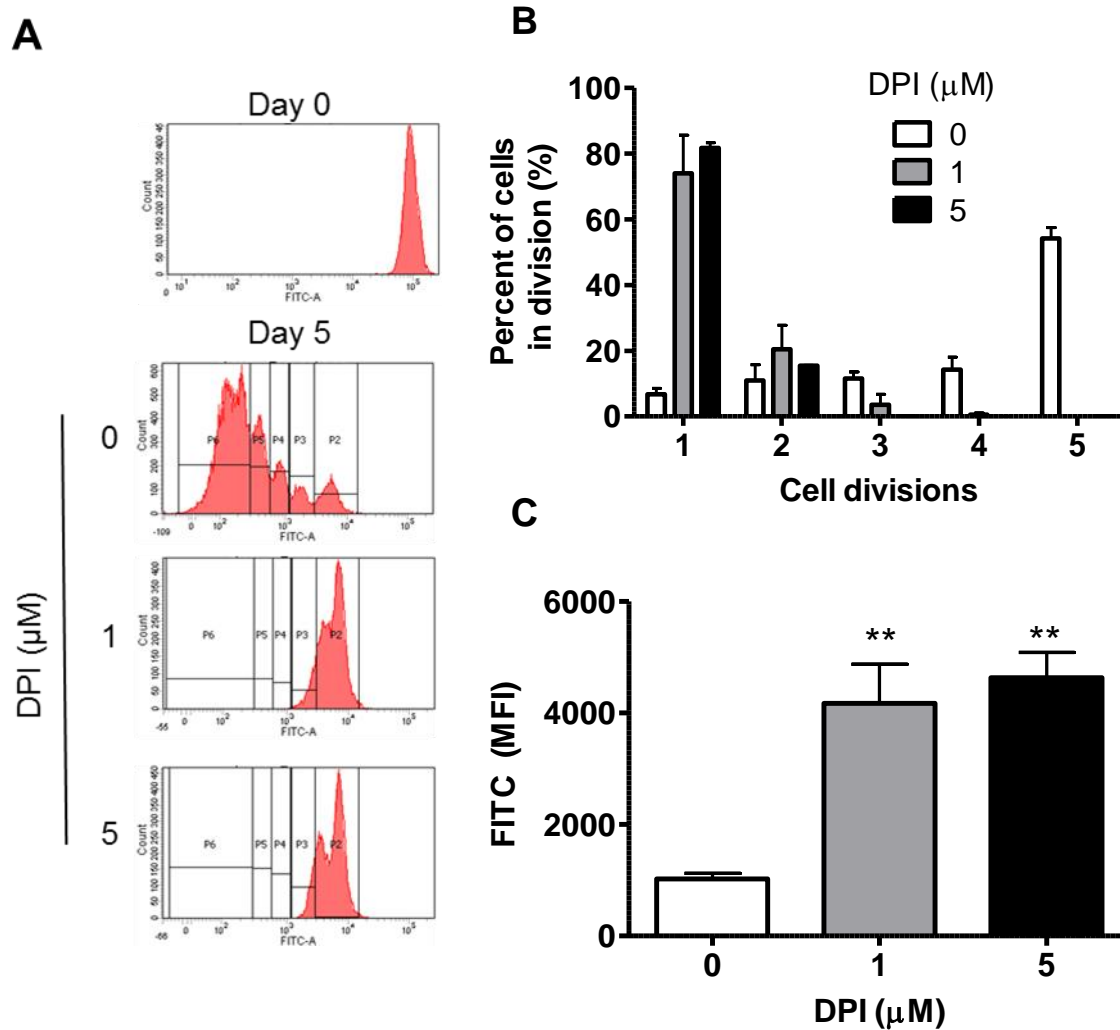


Figure 4.10 DPI inhibits TCR-induced proliferation of naïve T lymphocytes. Freshly isolated naïve CD4⁺ T lymphocytes were labelled with CFSE. 1 million cells were analysed on the same day (Day 0) or pre-treated with vehicle (media) or increasing concentrations of DPI for 30 minutes, followed by addition of anti-CD3/CD23 antibody coated beads to cells at a ratio of 3:1. IL-2 was added at a final concentration of 36 Units/ml and cells cultured for 5 days before analysis of CFSE fluorescence. Data are (A) representative FACS plots from one donor, (B) mean \pm SEM percentage of cells in each cell division and (C) CFSE mean \pm SEM fluorescence intensity from three independent donors. Statistical significance was determined by one way-ANOVA with Dunnett's post-test where ** $p < 0.01$ compared to control.

4.4.4 H₂O₂ had no effect upon SEB-activated T lymphocyte adhesion or integrin receptor expression

Ligation of the TCR is known to increase adhesion of lymphocytes (Simonson *et al.*, 2006). The adhesion of lymphocytes to components of the extracellular matrix and other cells is critical for cell migration, extravasation and formation of immunological synapses. However, the roles of ROS in T lymphocyte adhesion are not well established and were therefore examined.

Firstly, UCHT-1 (TCR agonist) significantly enhanced SEB-activated T lymphocyte adhesion to fibronectin (Figure 4.11 A). SEB-activated T lymphocytes were pre-treated with H₂O₂ for 30 minutes and allowed to adhere to fibronectin for 30 minutes. The plate was turned upside down for 15 minutes and the wells washed with PBS. Adherent cells were scraped and counted by flow cytometry. Interestingly, H₂O₂ had no effect upon basal or UCHT-1-induced adhesion to fibronectin (Figure 4.11 B). H₂O₂ also had no effect upon the expression of the α -subunit of integrin receptors LFA-1 (CD11a subunit) or α 4 β 1 (CD49 subunit) (Figure 4.11 C & D).

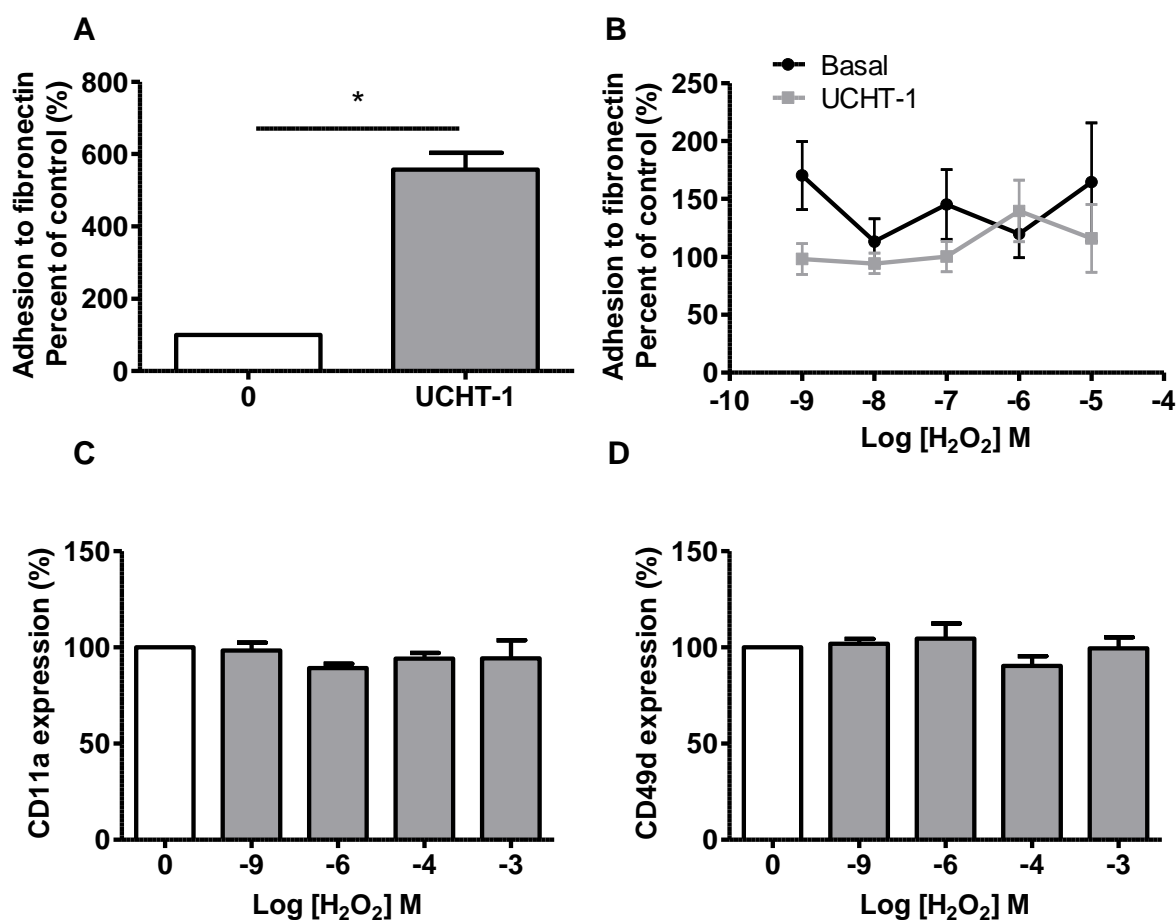


Figure 4.11 H₂O₂ has no effect upon CD11a, CD49d integrin expression and T lymphocyte adhesion to fibronectin. 1 million SEB- activated T lymphocyte (8-12 days post isolation) were treated with vehicle or increasing concentrations of H₂O₂ for 30 minutes. Cells were stimulated with UCHT-1 (10µg/ml) for 5 minutes, before being allowed to adhere to a fibronectin (10µg/ml) coated 96 well plate for 30 minutes. The plate was sealed and turned upside down for 15 minutes. Wells were washed gently with PBS to remove any un-adhered cells. Adhered cells were scrapped and counted using flow cytometry. (A) Indicates the adhesion observed with UCHT-1 (10µg/ml stimulation) compared to control. Statistical significance was determined by Students T test and where *p<0.05 as compared to control. (B) Data are represented as percent change from basal conditions. (C & D) Cells were treated with increasing concentrations of H₂O₂ for 30 minutes and then expression of the cell surface receptors (C) CD11a and (D) CD49d was assessed using PE-conjugated antibodies and analysed by flow cytometry. Data are represented as percent change from basal conditions and is the mean ± SEM of three independent experiments. Statistical significance was determined by one-way ANOVA with Dunnett's post-test.

4.5 Effect of ROS manipulation upon chemoattractant-induced responses

H₂O₂ has been observed to be an early danger cue required for neutrophil recruitment to wounds in zebrafish (Niethammer *et al.*, 2009) and is required for successful mouse T lymphocyte migration towards a chemoattractant (Hara-Chikuma *et al.*, 2012). To date, little is known about whether hydrogen peroxide is required for the migration of human T lymphocytes. Therefore the functional roles of ROS and antioxidants on human T lymphocytes migration were investigated.

4.5.1 H₂O₂ is not a chemoattractant for SEB-activated T lymphocyte migration

To determine whether H₂O₂ acts as a chemoattractant to SEB-activated T lymphocytes, an *in vitro* chemotaxis assay, manufactured by Neuroprobe, was utilised. SEB-activated T lymphocytes were placed above a porous membrane and the number of cells towards a chemoattractant in a bottom well counted. The effect of ROS were initially investigated in SEB-activated T lymphocytes as effector cells are more likely to be exposed to the high levels ROS present within inflamed or wounded tissues. Firstly, CXCL11 chemokine was used to validate the assay. The migratory response towards CXCL11 was bell shaped, with an optimal increase of 16.3 ± 3.7 fold occurring at 10nM CXCL11 (Figure 4.12 A).

Next, to determine whether H₂O₂ could also act as a chemoattractant, H₂O₂ was placed in the **bottom well** of the migration plate, indicated in schematic of Figure 4.12 B. H₂O₂ caused a concentration dependent decrease in SEB-activated T lymphocytes migration towards the lower well as compared to media alone. Migration was significantly impaired at 10µM H₂O₂ and by 1mM almost completely inhibited (Figure 4.12 C) with an IC₅₀ of 2.5µM (Figure 4.12 D). Interestingly, instead of acting like a chemoattractant H₂O₂ has suppressed the migration of human T lymphocytes

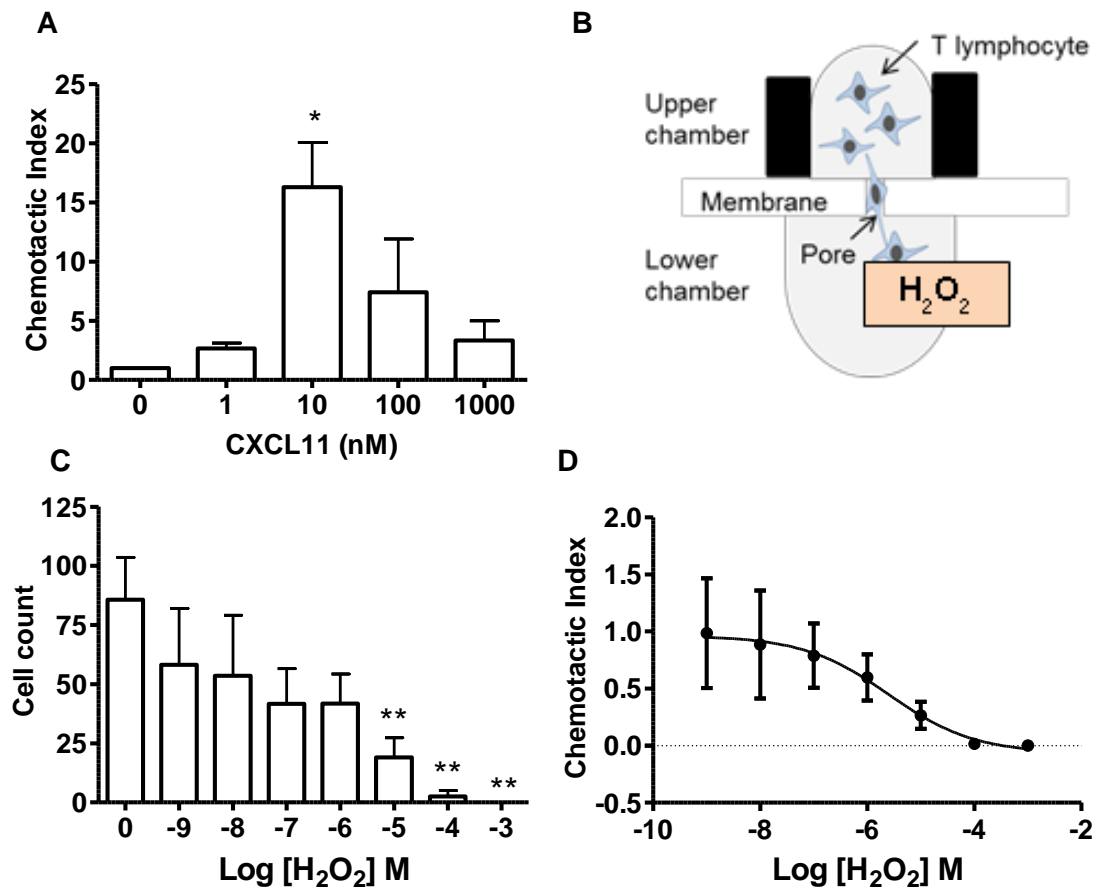


Figure 4.12 H_2O_2 does not act as a chemoattractant instead inhibits basal migration of SEB-activated T lymphocytes. SEB-activated T lymphocytes (8-12 days post isolation) were washed in serum free media and resuspended at 3.2 million per ml. Cell migration towards increasing concentrations of (A) CXCL11 or (C & D) H_2O_2 was then assessed by a Neuroprobe chemotaxis assay whereby cells are placed on top of a $5\mu m$ porous membrane and H_2O_2 placed in the bottom chamber. The number of cells migrated into the bottom well of were counted flow cytometry. (B) (A) Schematic illustrating the experimental set-up for data generated in C & D, to highlight that H_2O_2 was placed into the bottom well. Data are (C) Number of migrated cells and (B) Chemotactic index with a sigmoidal curve fit using graph Pad Prism 5. Data are mean \pm SEM from three independent donors. Statistical significance was determined using a one-way ANOVA with Dunnett's post-test where * $p < 0.05$ ** $p < 0.01$ compared to control.

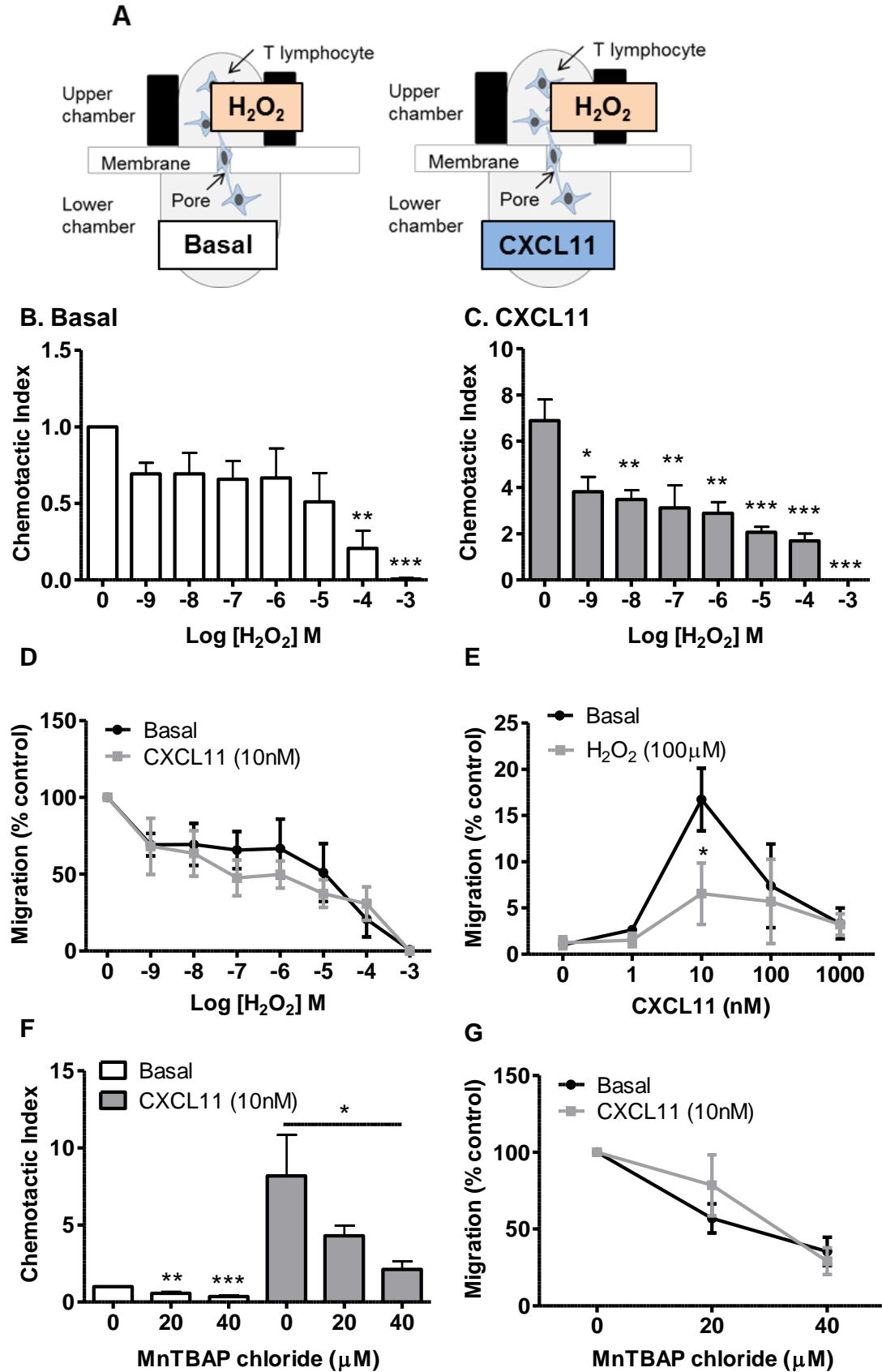
4.5.2 H₂O₂ inhibits SEB-activated T lymphocyte migration to the inflammatory chemokine CXCL11

Given that exogenous H₂O₂ previously appeared to impair basal migration of T lymphocytes (Figure 4.12), T lymphocytes were **pre-treated directly with H₂O₂** to further examine the impact of ROS levels upon basal and CXCL11-induced migration (Figure 4.13). Following antigen encounter, the chemokine receptor CXCR3 is up-regulated on T lymphocytes and is crucial for their recruitment into inflamed tissues (Cole *et al.*, 1998; Marwick *et al.*, 2010).

Basal migration was reduced by between 30-45% across the nanomolar and micromolar concentration range by pre-treatment with H₂O₂, although a significant inhibition of 80±1% was not observed until a concentration of 100µM was reached (Figure 4.13 B). These results were analogous to Figure 4.12 where H₂O₂ was present in the lower well and acted to suppress migration. However, excitingly CXCL11-induced SEB-activated T lymphocyte migration was significantly reduced across the whole range of H₂O₂ concentrations tested (Figure 4.13 C & D). 1nM H₂O₂ suppressed migration by 44.8 ± 4% of control conditions and completely inhibited CXCL11-induced migration at 1mM H₂O₂. As T lymphocytes are known to respond to CXCL11 in a bell-shaped fashion, the sensitivity of SEB-activated T lymphocytes towards increasing concentrations of CXCL11 was examined following pre-treatment with 100µM H₂O₂. Maximal migration occurred at 10nM for both vehicle control and H₂O₂ pre-treated cells, suggesting H₂O₂ does not alter T lymphocyte sensitivity to CXCL11 (Figure 4.13 E). Maximal migration for the H₂O₂ pre-treated cells was still significantly lower than the control cells.

4.5.3 MnTBAP chloride inhibits CXCL11-dependent SEB-activated T lymphocyte migration

Cells were directly pre-treated with MnTBAP chloride for 30 minutes, in order to increase intracellular H₂O₂ levels, prior to being plated in the cell migration assay and exposed to either basal media or CXCL11. T lymphocyte chemotaxis towards CXCL11 was significantly impaired to 29 ± 8.6 % of CXCL11 control when treated with 40 µM MnTBAP chloride. MnTBAP chloride also caused a significant, concentration dependent inhibition of basal SEB lymphocyte migration, with basal T lymphocyte migration reduced to 35 ± 9 % of control by 40 µM MnTBAP chloride (Figure 4.13 F & G). Increasing intracellular H₂O₂ levels therefore had a similar effect on SEB-activated T lymphocyte migration to exposure to exogenous H₂O₂ (Figure 4.13 B, C & D).



resuspended at 3.2 million cells per ml. Cells were pre-treated with either vehicle or increasing concentrations of (B, C & D) H_2O_2 for 30 minutes. The (B) basal migration of cells and (C) migration towards the chemokine CXCL11 (10nM) was then assessed by Neuroprobe assay. Data are expressed as chemotactic index where the number of cells migrated with each drug condition is divided by the vehicle control. (D) Data are shown as percentage of control migration. (E) Cells were pre-treated with 100 μM H_2O_2 for 30 minutes. Migration was then determined towards increasing concentrations of CXCL11. (F & G) Cells were pre-treated with MnTBAP chloride for 30 minutes. The basal and CXCL11 (10nM) migration was then assessed by Neuroprobe assay. Data are expressed as (F) chemotactic index and (G) percentage of vehicle control. Data are the mean \pm SEM of at least 3 independent experiments and statistical significance was determined by one-way ANOVA with Dunnett's post-test where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to control.

4.5.4 Dampening the redox environment upon SEB-activated T lymphocyte basal and CXCL11-induced migration

As application of exogenous H_2O_2 and using a SOD mimetic to increase intracellular H_2O_2 both inhibited migration, the effect of dampening H_2O_2 levels on T lymphocyte migration was examined. Two approaches were utilised; degradation of H_2O_2 by catalase and inhibition of superoxide production, by DPI. Cells were directly pre-treated with catalase for 30 minutes prior to being placed in the Neuroprobe assay. Catalase pre-treatment of SEB-activated T lymphocytes caused a concentration dependent enhancement of basal migration, with 1mg/ml catalase increasing control migration by 3.8 ± 0.6 fold. Catalase pre-treatment also significantly enhanced CXCL11-induced migration. CXCL11 alone increased migration by 8.9 ± 1.4 , which was enhanced to 20.1 ± 3.8 fold with the addition of 1mg/ml catalase (Figure 4.14 A & B). To control for possible impurities in the catalase solution, catalase was boiled for 10 minutes to heat-inactivate the enzyme. Heat-inactivated catalase had no effect upon cell migration (Figure 4.14 C). Catalase significantly enhancing both basal and chemokine migration suggests there is a source of ROS in both basal and CXCL11-induced T lymphocytes that impedes their migration.

Inhibition of superoxide production by DPI had no significant effect upon SEB T lymphocyte basal or CXCL11-induced migration (Figure 4.14 D & E). This suggests that the ROS that appears to be impeding migration in the catalase experiments is not generated by NOX enzymes.

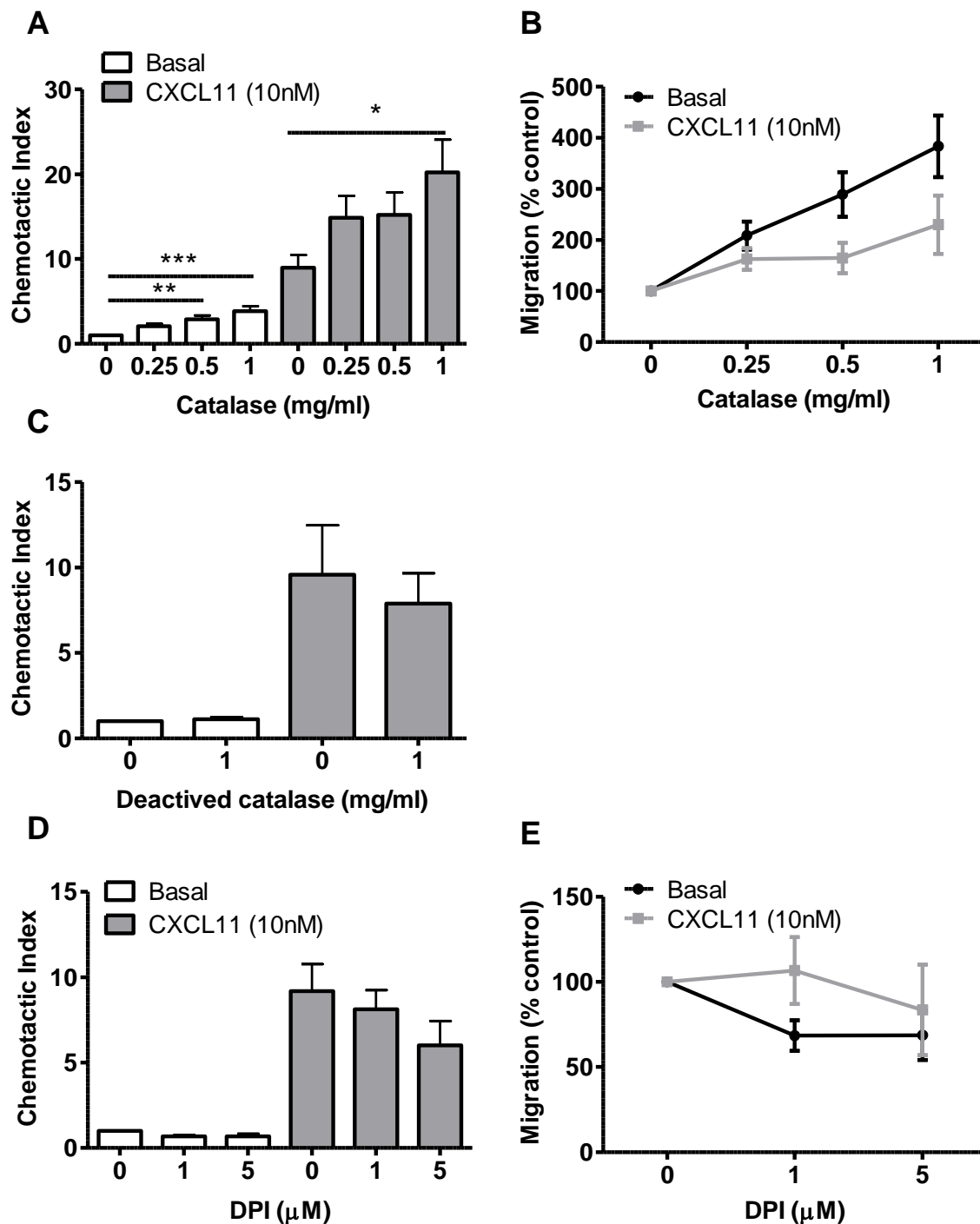


Figure 4.14 Catalase enhanced and DPI had no effect upon basal and CXCL11-induced SEB-activated T lymphocyte migration. SEB-activated T lymphocytes (8-12 days post isolation) were washed in serum free media and resuspended at 3.2 million cells per ml. Cells were pre-treated with either vehicle or stated concentrations of (A, B) catalase, (C) deactivated catalase or (D, E) DPI for 30 minutes. The basal migration and migration towards the chemokine CXCL11 (10nM) of SEB-activated T lymphocytes was then assessed by Neuroprobe assay. Data are expressed as chemotactic index or as percentage of control migration of at least three independent donors, with samples performed in duplicate. Data are displayed as the mean \pm SEM and statistical significance was determined by one-way ANOVA with Dunnett's post-test where ** $p < 0.01$ and *** $p < 0.001$ compared to control.

4.5.5 Hypoxia significantly impairs SEB-activated T lymphocyte migration

Inflammation is known to produce a highly hypoxic environment due to high cell recruitment and enhanced cellular activity causing large alterations in oxygen supply and demand, which ultimately results in cellular exposure to higher levels of ROS. SEB-activated T lymphocytes will be exposed to hypoxia and increased ROS levels when migrating to an inflammatory environment due to the high number of active cells present. The effect of exposing migrating SEB-activated T lymphocytes to a hypoxic environment was therefore examined to assess whether this model would have a similar effect as pharmacologically increasing ROS levels. The migration of SEB-activated T lymphocytes towards CXCL11 was examined in “hypoxia” (5% O₂, 5% CO₂, 90% N₂ 37°C) and compared to cells migrating under standard cell culture conditions or “normoxia” (air [approximately 21% O₂ & 78% nitrogen]: 5% CO₂).

SEB-activated T lymphocyte migration toward CXCL11 was impaired in cells incubated in 5% O₂. Chemotactic migration was reduced across the whole range of CXCL11 concentrations tested, and significantly suppressed to 53.7±18.6% of control migration at 100nM CXCL11 (Figure 4.15 A). T lymphocytes normally respond to CXCL11 in a bell shaped fashion, however both control and hypoxic treated cells appeared to have a concentration dependent response in this experiment.

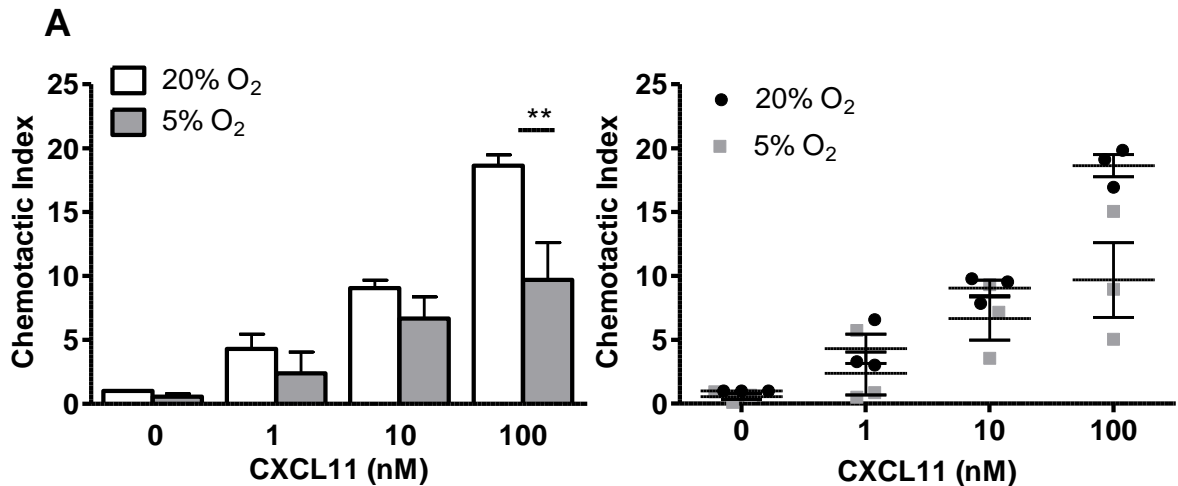


Figure 4.15 Hypoxia appears to suppress SEB-activated T lymphocyte migration. SEB-activated T lymphocytes (8-12 days post isolation) were washed in serum free media and resuspended at 3.2 million cells ml. Cells were incubated in either standard cell culture conditions as a control (air: 5% CO₂: 37°C), (A) 5% O₂, 5% CO₂, 90% N₂ 37°C (n=3) hypoxic chamber for 1 hour. The basal and CXCL11-induced (1-100nM) migration was then assessed by Neuroprobe assay. Data are expressed as the chemotactic index and are mean ± SEM of (A) three independent donors and (B) Three independent donors, with the right panel showing the individual donor responses. Statistical significance was determined by two-way ANOVA with Tukey's post-test where **p<0.01 as compared to standard cell conditions control.

4.6 H₂O₂ has no effect upon SEB-activated T lymphocyte migration to the inflammatory chemokine CXCL10 or homeostatic chemokine CXCL12

As H₂O₂ significantly impaired migration towards CXCL11, its effect upon chemotactic migration to alternative chemokines was investigated. Two chemokines were utilised, an inflammatory chemokine CXCL10, which also binds to CXCR3, and a homeostatic chemokine CXCL12 which binds to CXCR4 (Figure 4.16 A). In order to properly control this experiment, the effect of H₂O₂ upon CXCL11-induced migration was re-examined to eliminate variability between previous experiment being undertaken on different donors and at a different time.

Although CXCL11 and CXCL10 are both induced by IFN γ and thought to promote Th1 immune responses (Luster *et al.*, 1985; Farber, 1990; Cole *et al.*, 1998), they have previously been shown to have distinct expression patterns, potencies and efficacies in a number of assays, including internalisation and migration (Xanthou *et al.*, 2003; Colvin *et al.*, 2006; Dagan-Berger *et al.*, 2006). CXCL11 significantly increased SEB-T lymphocyte migration 6.9 ± 0.9 fold over basal migration, whereas CXCL10 was less potent and migration was increased 3.1 ± 0.9 fold over basal migration (Figure 4.16). The observation of CXCL10 and CXCL11 exhibiting distinct efficacies to CXCR3 led to the suggestion that they interact with CXCR3 in different ways and are likely to stabilize different conformations of the receptor (Nedjai *et al.*, 2012). Consistently with previous results (Figure 4.12), H₂O₂ (1nM) significantly impaired CXCL11 migration (Figure 4.16 B). However interestingly, only toxic concentration of H₂O₂ (10mM) inhibited CXCL10-induced migration (Figure 4.16 C). CXCL12 increased migration 5.7 ± 1.1 fold over basal. However, similarly to CXCL10, only H₂O₂ (10mM) significantly inhibited migration toward CXCL12 (Figure 4.16 D) and this is likely to be due to toxicity. This observation is really interesting and suggests H₂O₂ is specifically altering CXCL11 induced migration even though it acts through the same receptor as CXCL10.

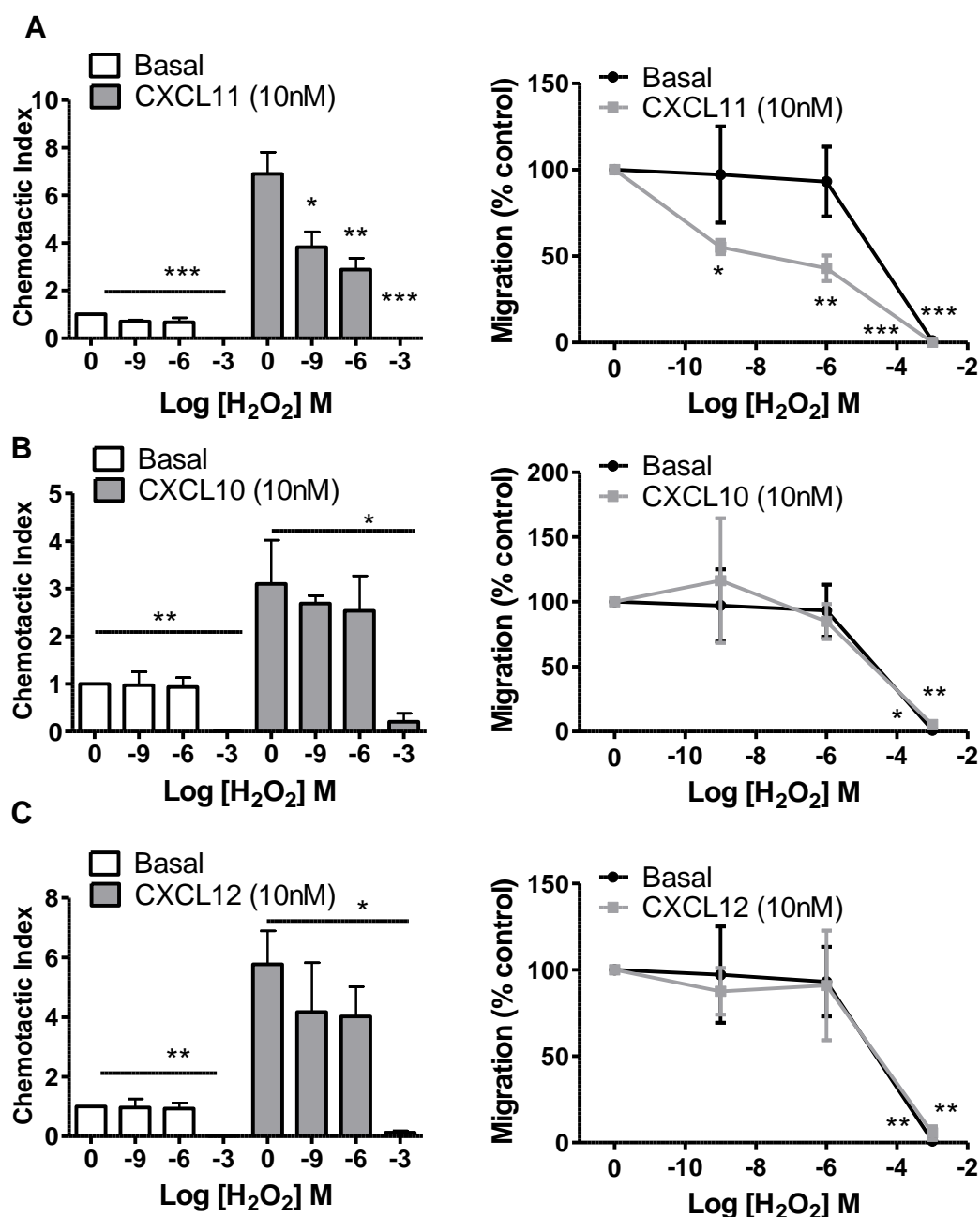


Figure 4.16 CXCL10 and CXCL12-induced migration of SEB-activated T lymphocytes is inhibited by high concentrations of H₂O₂. SEB-activated T lymphocytes (8-12 days post isolation) were washed in serum free media and resuspended at 3.2 x million cells per ml. Cells were pre-treated with either vehicle or stated concentrations of H₂O₂ for 30 minutes. The basal migration of cells and migration towards the chemokine (A) CXCL11 (10nM), (B) CXCL10 (10nM) or (C) CXCL12 (10nM) was then assessed by Neuroprobe assay. Data are expressed as chemotactic index (Left panel) or as percentage of control migration (Right panel). Data are the mean \pm SEM of three independent donors. Statistical significance was determined using a one-way ANOVA with Dunnett's post-test where * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to control.

4.7 ROS manipulation on naïve T lymphocyte migration

Given that ROS manipulation alters the migratory responses of SEB-activated T lymphocytes to CXCL11, the effect of increasing or decreasing ROS levels was examined on naïve T lymphocytes. CXCR4 is highly expressed on naïve T lymphocytes and therefore their migration to CXCL12 was examined.

4.7.1 Low concentrations of H₂O₂ inhibited basal migration of Naïve T lymphocytes

Naïve T lymphocytes were treated with H₂O₂ for 30 minutes before being placed in the migration assay and exposed to either basal media or CXCL12 in the bottom well. Similarly to the SEB-activated T lymphocytes (Figure 4.13), basal naïve T lymphocyte migration was significantly inhibited at concentrations greater than or equal to 1μM H₂O₂ (Figure 4.17 A).

Although a concentration dependent suppression of migration was observed across the micromolar and millimolar concentration range of H₂O₂, only 1mM H₂O₂ caused a significant inhibition of CXCL12-induced migration (Figure 4.17 B). Naïve T lymphocyte migration appeared to be more resistant to exogenous H₂O₂ pre-treatment as 1mM pre-treatment of H₂O₂ completely attenuated migration of the SEB- activated T lymphocytes (Figure 4.13), whereas 35 ± 8 % of control naïve T lymphocytes still migrated.

To examine the impact of increased ROS levels in naïve T lymphocytes were pre-treated with MnTBAP chloride for 30 minutes prior to being plated in the cell migration assay and exposed to either basal media or CXCL12. Unlike in the SEB-activated T lymphocytes, naïve T lymphocyte basal and CXCL12-induced migration was not significantly altered when treated with up to 40 μM MnTBAP chloride (Figure 4.17 C & D). This could be due to MnTBAP chloride only marginally increasing levels of H₂O₂ and naïve T lymphocytes migration being more resistant to H₂O₂ concentrations.

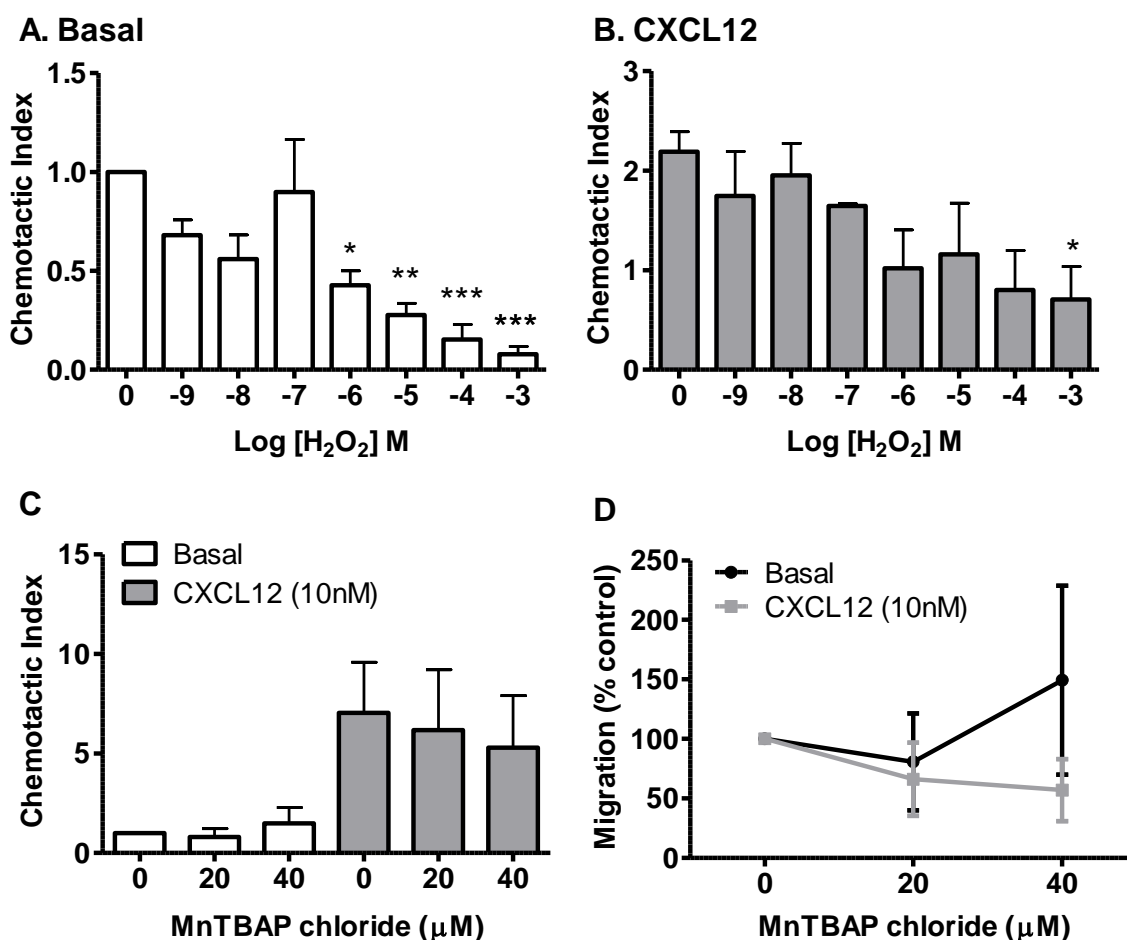


Figure 4.17 Effect of H₂O₂ and MnTBAP chloride upon basal and CXCL12-induced Naïve CD4 T lymphocyte migration. Naïve CD4 T lymphocytes were washed in serum free media and resuspended at 3.2 million cells per ml. Cells were pre-treated with either vehicle or increasing concentrations of (A & B) H₂O₂ for 30 minutes. The (A) basal migration of cells and (B) migration towards the chemokine CXCL12 (10nM) was then assessed by Neuroprobe assay. Data are expressed as chemotactic index, where the number of cells migrated with each drug condition is divided by the vehicle control. (C & D) Cells were pre-treated with MnTBAP chloride for 30 minutes. The basal and CXCL12 (10nM)-induced migration was then assessed by Neuroprobe assay. Data are expressed as (C) chemotactic index and (D) percentage vehicle control. Data are the mean \pm SEM of three independent donors, with samples performed in duplicate. Statistical significance was determined using a one-way ANOVA with Dunnett's post-test where * p <0.05 ** p <0.01 and *** p <0.001 compared to control.

4.7.2 Catalase enhanced but DPI had no effect upon basal and directional migration of naïve T lymphocytes

ROS levels were dampened in naïve T lymphocyte by exposure to catalase and DPI, and the impact upon migration examined. Consistent with activated T lymphocytes (Figure 4.14), catalase caused a concentration dependent enhancement of basal migration with 1mg/ml catalase increasing vehicle control migration by 12.5 ± 6.6 fold (Figure 4.18 A). Catalase pre-treatment also significantly enhanced CXCL12-induced migration. CXCL12 alone increases migration by 6.3 ± 3.4 fold, which was enhanced to 40.7 ± 10.34 fold following pre-treatment of 1mg/ml catalase (Figure 4.18 A). Similarly to SEB-activated T lymphocytes (Figure 4.14), DPI had no significant effect upon naïve T lymphocyte basal or CXCL12-induced migration (Figure 4.18 B).

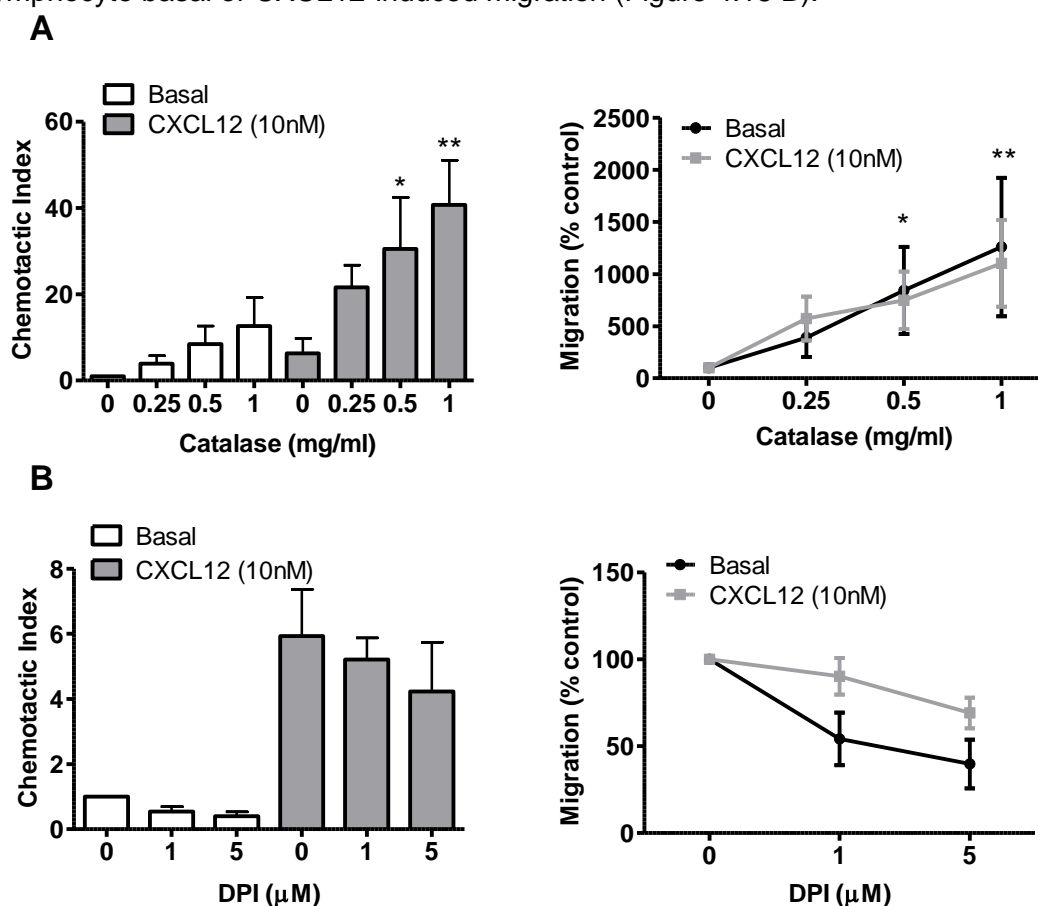


Figure 4.18 Effect of catalase and DPI upon basal and CXCL12-induced Naïve T lymphocyte migration. Naïve T lymphocytes were washed in serum free media and resuspended at 3.2 million cells per ml. Cells were pre-treated with either vehicle or increasing concentrations of (A) catalase or (B) DPI for 30 minutes. The basal migration of cells and migration towards the chemokine CXCL12 (10nM) was then assessed by Neuroprobe assay. Data are expressed as the mean \pm SEM of chemotactic index (Left panel) or percentage of control migration (Right panel) of at least three independent donors, with samples performed in duplicate. Statistical significance was determined by one-way ANOVA with Dunnett's post-test where * $p < 0.05$ and ** $p < 0.01$ compared to control.

4.8 Effect of H₂O₂ upon CXCL11-induced Ca²⁺ and actin regulation

4.8.1 H₂O₂ has no effect upon CXCL11 dependent Ca²⁺ signalling

Following CXCR3/CXCL11 stimulation, elevation of intracellular Ca²⁺ concentrations occurs. Elevated intracellular Ca²⁺ is required for directed cell migration as local Ca²⁺ pulses near the leading edge of the cell activate myosin and to modulate focal adhesions (Tsai *et al.*, 2014). Intracellular Ca²⁺ was measured to assess whether H₂O₂ affected CXCR3/CXCL11 signalling potential. SEB-activated T lymphocytes were loaded with a Ca²⁺ sensitive fluorescent reporter (Fluo-4), and Ca²⁺ dependent fluorescence measured every 15 seconds throughout the experiment. In Chapter 3, Figure 3.10, CXCL11-induced a concentration dependent, transient although small increase in intracellular Ca²⁺. Pre-treatment of SEB-activated T lymphocytes with increasing concentrations of H₂O₂ for 30 minutes had no effect upon CXCL11-induced Ca²⁺ at any of the concentrations studied (Figure 4.19), which suggests the defect in migration is independent of Ca²⁺ signalling.

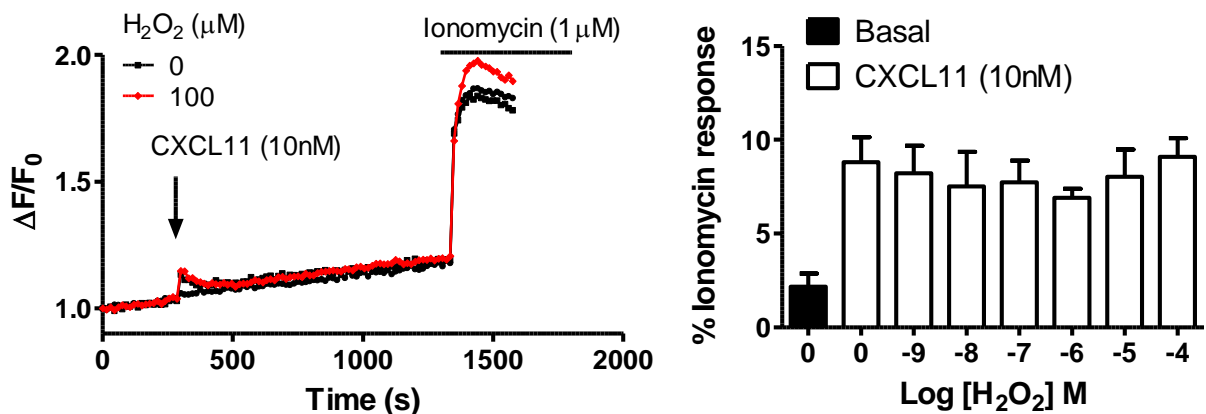


Figure 4.19 Pre-treatment with H₂O₂ had no effect upon CXCL11-induced intracellular Ca²⁺ elevation in SEB-activated T lymphocytes. SEB-activated T lymphocytes (8-12 days post isolation) were washed into PBS and loaded for 45 minutes with 1μM Fluo-4. Cells were washed 3 times and re-suspended at 5 million cells per ml in Ca²⁺ free-HBSS and extracellular Ca²⁺ adjusted to 1mM. Cells were plated in a black 96 well plate and either allowed to rest or treated with stated concentrations of H₂O₂ for 30 minutes. Fluorescence was recorded over time using a plate reader at 37°C. 10nM CXCL11 was added after a baseline was established and 1μM ionomycin was used as a loading control. Left panel is a kinetic trace indicating change in fluorescence from basal ($\Delta F/F_0$) from one representative donor. The right panel is the peak change in fluorescence generated by UCHT-1 as a percentage of the peak change generated by ionomycin and is the mean \pm SEM from averaged duplicates of three independent donors. Statistical significance was determined by one-way ANOVA with a Dunnett's post-test relative to control.

4.8.2 H₂O₂ inhibits T lymphocyte polarisation and enhances basal actin polymerisation

Preventing H₂O₂ uptake has been previously shown to impair actin polymerisation in murine T lymphocytes (Hara-Chikuma *et al.*, 2012) whilst oxidative stress enhances actin polymerisation and attenuates actin polarisation to CXCL11 in human naïve T lymphocytes (Klemke *et al.*, 2008). As high concentration of H₂O₂ impeded basal and chemokine-induced SEB-activated T lymphocyte migration (Figure 4.13 & 4.16), the importance of a high concentration of H₂O₂ in regulating morphological and structure changes required for migration was examined

Cells were treated with either H₂O₂ (100µM) or catalase (1mg/ml) for 30 minutes then fixed, permeabilized and stained with TRITC tagged phalloidin. Mean fluorescence intensity of TRITC fluorescence was determined using flow cytometry. Interestingly, H₂O₂ significantly increased the level of polymerised F-actin detected to 195 ± 30% of control levels Catalase pre-treatment had no effect upon the level of polymerised F-actin (Figure 4.20).

Following the observation basal actin polymerisation was affected by 100µM H₂O₂ exposure (Figure 4.20), the effect of H₂O₂ on actin polarisation to CXCL11 was determined. In the absence of H₂O₂ cells showed a polarised F-actin distribution upon CXCL11 stimulation, however cells treated with H₂O₂ did not have polarised actin (Figure 4.21). This suggests that high concentrations of H₂O₂ could impair T lymphocyte migration by dysregulation cellular actin and inhibiting the ability of the cell to polarise to chemokine

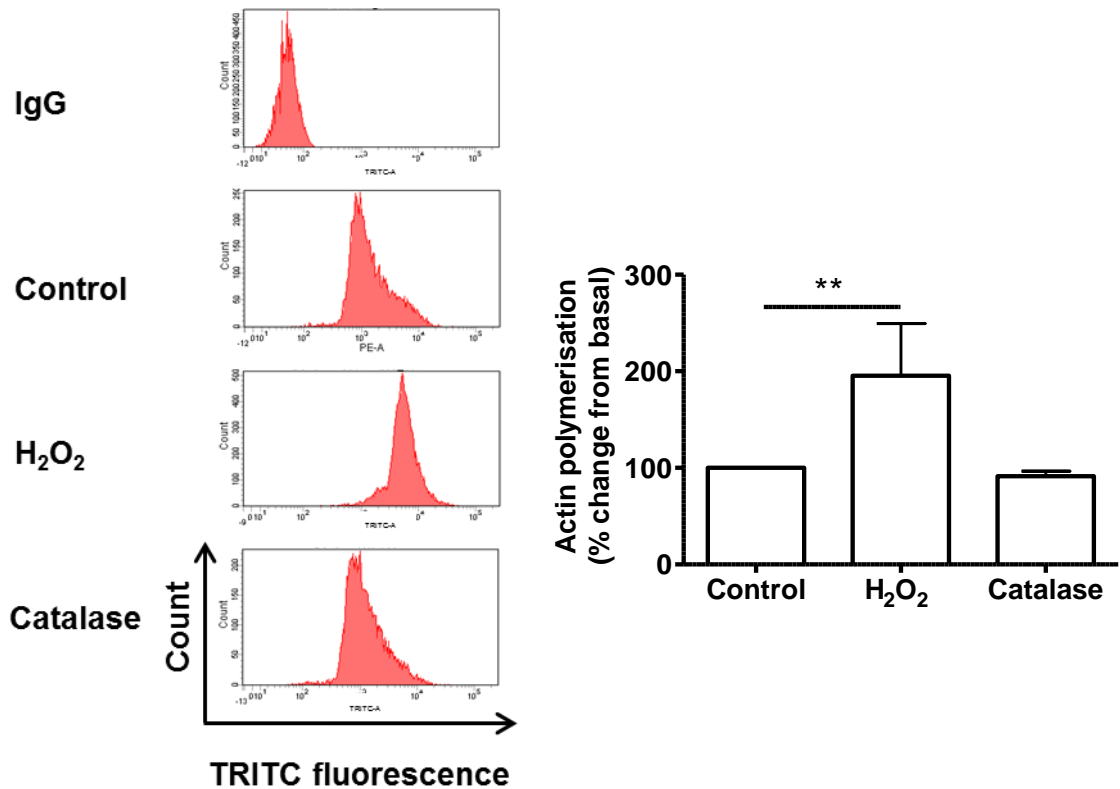


Figure 4.20 H₂O₂, but not catalase, increases actin polymerisation of SEB-activated T lymphocytes. 1 million SEB-activated T lymphocytes (8-12 days post isolation) were washed three times in serum free media and treated with H₂O₂ (100μM) or catalase (1mg/ml) for 30 minutes. Cells were fixed using BD fixation reagent for 20 minutes, permeabilized and incubated with TRITC tagged phalloidin for 30 minutes. Mean fluorescence intensity per 10,000 cell was measured using flow cytometry. Data are (left panel) representative FAC plots from one donor and (right panel) mean ± SEM minus IgG control, normalised to the untreated control from three independent donors. Statistical significance was determined by one way-ANOVA with Dunnett's post-test where **p<0.01 compared to control.

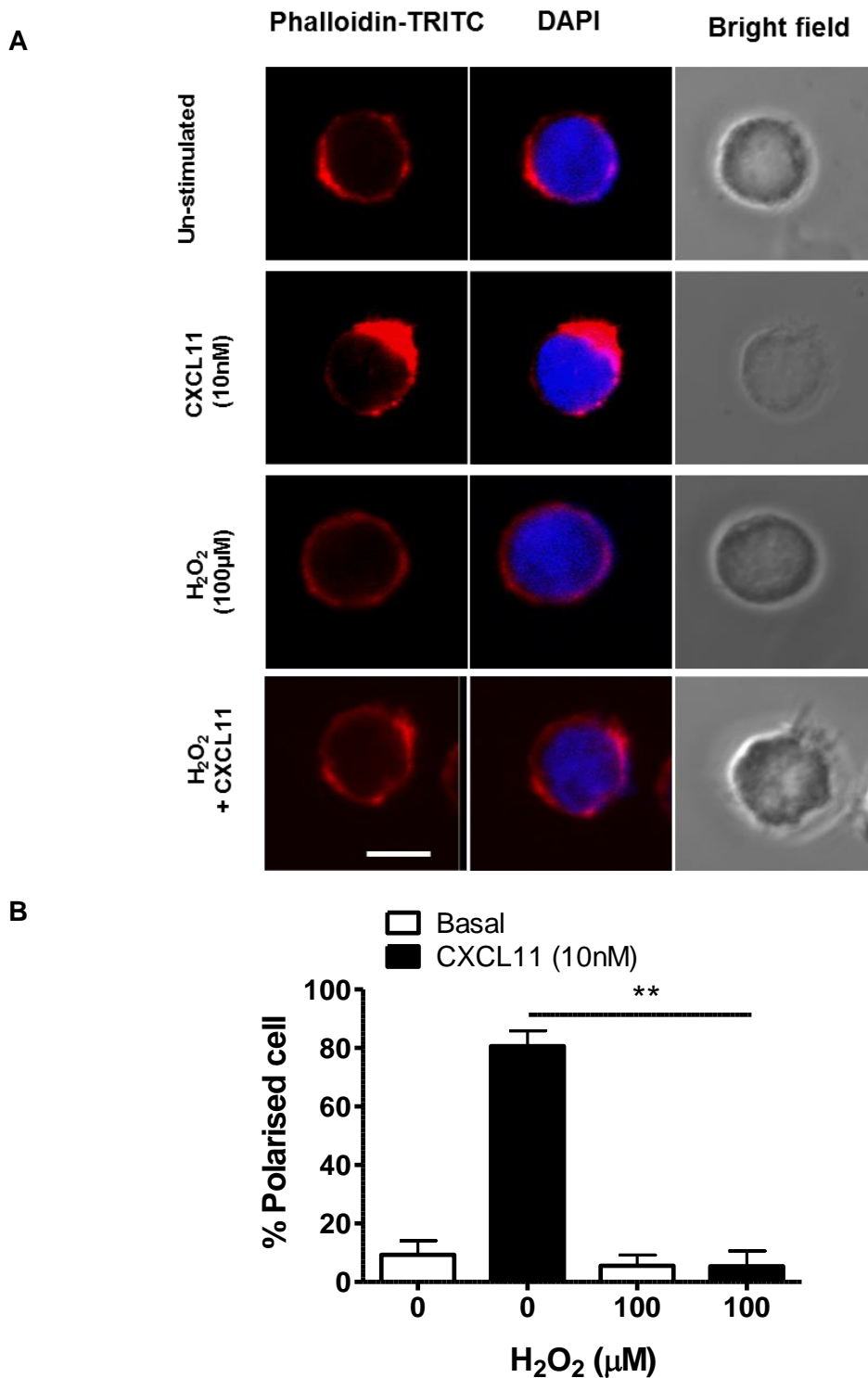


Figure 4.21 Effect of H₂O₂ and CXCL11 upon polarisation of SEB-activated T lymphocytes. 1 million SEB-activated T lymphocytes (8-12 days post isolation) were pre-treated with 100μM H₂O₂ for 30 minutes then stimulated with 10nM CXCL11 for 5 minutes. Then cells were fixed in BD fixation reagent, permeabilised with BD permeabilisation reagent and stained with TRITC tagged phalloidin and DAPI. (A) Representative images showing actin and DAPI staining. The scale bar represents 10μm. (B) Represents the mean ± SEM percentage of polarised cells observed from three independent donors, with 20 cells counted for each condition. Statistical significance was determined by two-way ANOVA with a Bonferroni post-test where **p<0.01 compared to control.

4.9 Chapter 4 - Results Summary

(a) TCR responses:

- Extracellular ROS is required for long term SEB-activated T lymphocyte survival
- H₂O₂ pre-treatment had no effect upon TCR-induced Ca²⁺ elevation
- H₂O₂ significantly inhibited CD3/CD28-induced proliferation of naïve T lymphocytes
- MnTBAP chloride had no effect upon CD3/CD28-induced proliferation of naïve T lymphocytes
- DPI and catalase significantly impaired T lymphocyte proliferation
- Basal and UCHT-1 stimulated adhesion to fibronectin was unaffected by H₂O₂ pre-treatment
- H₂O₂ had no effect upon expression of integrin receptor CD11a and CD49d

(b) Migration and chemokine-induced responses:

- H₂O₂ did not act as a chemoattractant for SEB-activated T lymphocytes
- Exogenous H₂O₂ and SOD dismutase mimetic inhibits basal and CXCL11-induced migration
- Catalase enhances basal and CXCL11-induced SEB-activated T lymphocyte migration
- DPI has no effect upon SEB-activated T lymphocyte migration
- H₂O₂ had a less potent effect upon CXCL10 and CXCL12-induced migration of SEB-T lymphocytes
- Naïve T lymphocyte migration was less potently effected by H₂O₂ pre-treatment whilst MnTBAP chloride and DPI had no effect; however catalase significantly enhanced basal and CXCL12-stimulated migration
- High concentrations of H₂O₂ induce F-actin polymerisation and inhibits F-actin polarisation to CXCL11
- H₂O₂ had no effect upon CXCL11-induced Ca²⁺ elevation

4.10 Chapter 4 Discussion

Validation of ROS manipulators - DCF fluorescence

Oxidative sensitive dyes such as DCF are widely used to determine the levels of oxidants within cells. However, it is important to note the DCF can be oxidised by multiple species of oxidants (Zurgil *et al.*, 2006), the actual species of oxidants produced in T lymphocytes have yet to be described. H_2O_2 was used as a positive control to verify DCF activity and confirm that treatment with exogenous H_2O_2 gives rise to a pro-oxidant intracellular environment. As the pharmacological antioxidants catalase and superoxide dismutase mimetic MnTBAP chloride are used to assess the roles of ROS in T lymphocytes, their activity was verified using DCF fluorescence. The superoxide dismutase mimetic enhanced the oxidation of DCF whilst catalase significantly reduced ROS production, confirming that these were suitable agents for manipulating ROS level in SEB-activated T lymphocytes. ROS producers and antioxidant defence mechanisms are illustrated in Figure 1.4. The compounds used in this chapter are summarised in Figure 4.1. To further this work other compounds could have been investigated which also manipulate cellular and extracellular ROS. This approach induces 1) cell penetrating recombinant catalase, 2) extracellular SOD dismutase, 3) apocynin which inhibits NADPH oxidase, 4) antimycin A, which inhibits cytochrome C-reductase and evokes the production of toxic quantities of free radical super oxide and 5) rotenone which inhibits the transfer of electrons from complex I to ubiquinone which creates a back-log of electrons in the mitochondrial matrix and enhanced superoxide production (Figure 4.22). In particular it would have been interesting to determine the effect of these reagents on SEB-activated T lymphocyte migration.

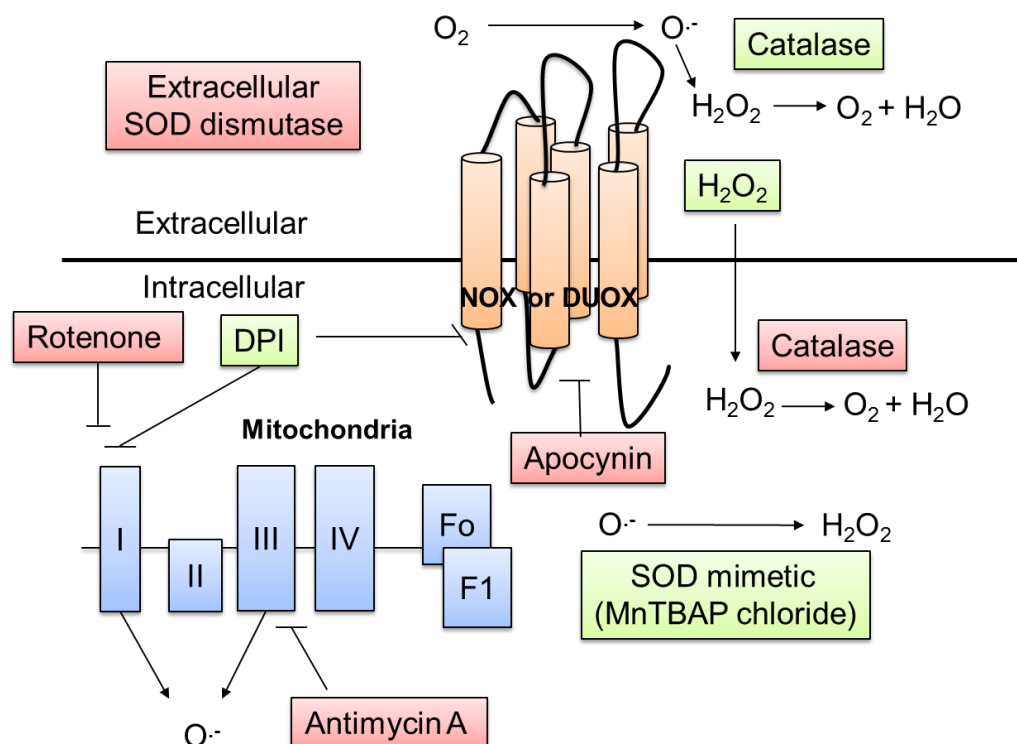


Figure 4.22 Additional pharmacological manipulators of ROS. Schematic highlighting in red boxes some additional pharmacological compounds which could be used to manipulate ROS within cells. These reagents could further the observations reported in this chapter.

The effect of ROS upon T lymphocyte viability

ROS species are known to induce bifurcate responses, with high concentrations evoking cell death and low concentration required for cell survival. H_2O_2 can be converted to highly reactive $\cdot OH$ radicals or reactive metal-oxygen intermediates which are cytotoxic (Halliwell *et al.*, 1990). Most experimental models, including those used in this chapter, use a bolus addition of H_2O_2 to the culture media. However, the cytotoxic concentrations of H_2O_2 reported in the literature are variable across different cell types and appear to be affected by exposure time and intracellular concentration (Gulden *et al.*, 2010). For example, naïve mouse T lymphocytes are reported to undergo oxidative stress at concentrations above $10\mu M$ (Klemke *et al.*, 2008), whereas the T lymphoblast cell line Jurkat are significantly affected at concentrations above $50\mu M$ (Cox 2007). In mammalian cells, cytotoxic concentrations of H_2O_2 have been reported between 10 - $1000\mu M$ (Nakamura *et al.*, 2003).

Here, to determine the cytotoxic concentration of H_2O_2 in human peripheral activated T lymphocytes, cell viability was assessed using two distinct approaches; (1) MTT assay to determine the effect upon mitochondrial activity and (2) propidium iodide and annexin-V staining to investigate whether H_2O_2 -induced cellular apoptosis or necrosis. The MTT assay verified that cells treated with $100\mu\text{M}$ H_2O_2 were still viable after 3 hours. However 24 hour treatments suggests that long term exposure to $10\mu\text{M}$ H_2O_2 will significantly decrease survival. This implies that both concentration and exposure time of H_2O_2 can affect the survival of T lymphocytes. Therefore, throughout Chapters 4 and 5, the exposure time to H_2O_2 was strictly controlled and all pre-exposures of H_2O_2 were for 30 minutes. The literature also describes that T lymphocytes can also have distinct susceptibility to H_2O_2 depending upon their stage of development. Preferential cell death occurred in CD8+ effector memory T lymphocytes (CD45RA - CCR7 -) as compared to naïve (CD45RA+) (Takahashi *et al.*, 2005). This could explain why naïve T lymphocytes appear to be more resistant to high concentrations of H_2O_2 in the migration assays. However, the effect of ROS manipulators on naïve T lymphocytes was not studied here. It would be interesting to determine how long H_2O_2 lasts in cell culture before it diffuses or is degraded, thus whether cell death occurs due to a delayed cytotoxicity or due to prolonged exposure. This could be determined by washing away H_2O_2 after the initial exposure and then observing the effect on survival.

As the MTT assay is redox based, results could be have been skewed if ROS treatment were still present. Therefore, cell viability was also assessed using annexin V and propidium iodide staining. ROS has been observed to enhance apoptosis of T lymphocytes (Buttke, 1995) however H_2O_2 did not induce apoptosis in peripheral T lymphocytes. Interestingly, thymoquinone, a compounds that induces ROS, has been observed to selectively evoke apoptosis of malignant T lymphocytes (Dergarabetian *et al.*, 2013). PI staining confirmed enhanced necrosis of T lymphocytes but only at 10mM H_2O_2 concentrations. High level of H_2O_2 exposure has been previously shown to induce oxidative DNA lesions, lipid peroxidation and mitochondrial damage in mammal cells (Nakamura *et al.*, 2003).

To test whether low levels of ROS are required for long term survival of T lymphocytes, cell viability in the presence of anti-oxidants was examined by MTT and AV/PI staining. After a 3 hours exposure, all concentrations of catalase, MnTBAP chloride and DPI had no effect upon cell viability. Conversely, 24 hour treatment with catalase and DPI decreased activated T lymphocyte viability, indicating that ROS are required for long term survival. Interestingly, a new SOD mimetic isoform MnTBAP chloride has been shown to have specific cytotoxic properties against acute T lymphoblastic leukaemia

cells, and comparatively to our study, had no effect upon the healthy T lymphocyte viability (Pica *et al.*, 2015). This suggests that pharmacological manipulation of ROS levels could represent a novel therapeutic strategy of selectively targeting malignant T lymphocytes.

The role of ROS in TCR-induced proliferation and adhesion of human T lymphocytes

Over the last decade, it has been extensively observed that ROS have putative roles in a vast number of physiological processes, including TCR activation and signalling. In addition, (Jackson *et al.*, 2004) ligation of the TCR receptor leads to the generation of ROS in T lymphocytes (Devadas *et al.*, 2002; Kwon *et al.*, 2010; Hara-Chikuma *et al.*, 2012; Sena *et al.*, 2013).

(a) Proliferation

Naïve T lymphocyte proliferation was induced with anti-CD3 and anti-CD28 antibodies, which were immobilized on microbeads. Immobilized antigen is thought to be more physiologically representative as they mimic antigen presenting cells and allow the formation of focal adhesions (Arndt *et al.*, 2013). Moreover, a recent study has indicated that extracellular ROS produced on TCR stimulation is involved in cross talk between the T lymphocyte and antigen presenting dendritic cell to stabilise the activation (Angelini *et al.*, 2002; Hivroz *et al.*, 2012). The involvement of ROS in TCR-induced proliferation was therefore examined.

H₂O₂ caused a significant defect in the proliferation of CD3/CD28-stimulated naïve T lymphocytes but only at concentrations above 10µM. This was consistent with the concentrations of H₂O₂ that impaired T lymphocyte viability over 24 hours. MnTBAP chloride had no effect upon cell proliferation, which was consistent with it having no effect upon cellular viability, and with the effect of low concentrations of H₂O₂ on cellular proliferation. 10µM H₂O₂ had no effect upon TCR-induced Ca²⁺ production in SEB-activated T lymphocytes, thus Ca²⁺ regulation does not appear to be the mechanism by which H₂O₂ alters TCR-induced proliferation or survival.

However, actin rearrangement is known to be important in cell division with dysregulation causing failure of centrosome separation and spindle assembly in the S

phase of the cell cycle (Bendris *et al.*, 2015). High concentrations of H_2O_2 enhance F-actin production and inhibit actin polarisation to chemokines. Dysregulation of actin could also underlie the inability of T lymphocytes to proliferate. Furthermore, during the cell cycle, a number of checkpoints ensure that proliferation is regulated, in particular the G1 checkpoint and G1 to S phase transition, which in general prevents unhealthy cells from proceeding further through the cell cycle (Foster *et al.*, 2010). High levels of exogenous H_2O_2 induce Akt, c-Src and ERK phosphorylation in human T lymphocytes (Section 5.2). High levels of positive cellular signalling could indicate that the cell is unhealthy and in order to limit the chance of developing leukaemias these cells are prevented from proliferating. The possible reasons that high concentrations of H_2O_2 inhibited human T lymphocyte proliferation are summarised in Figure 4.23

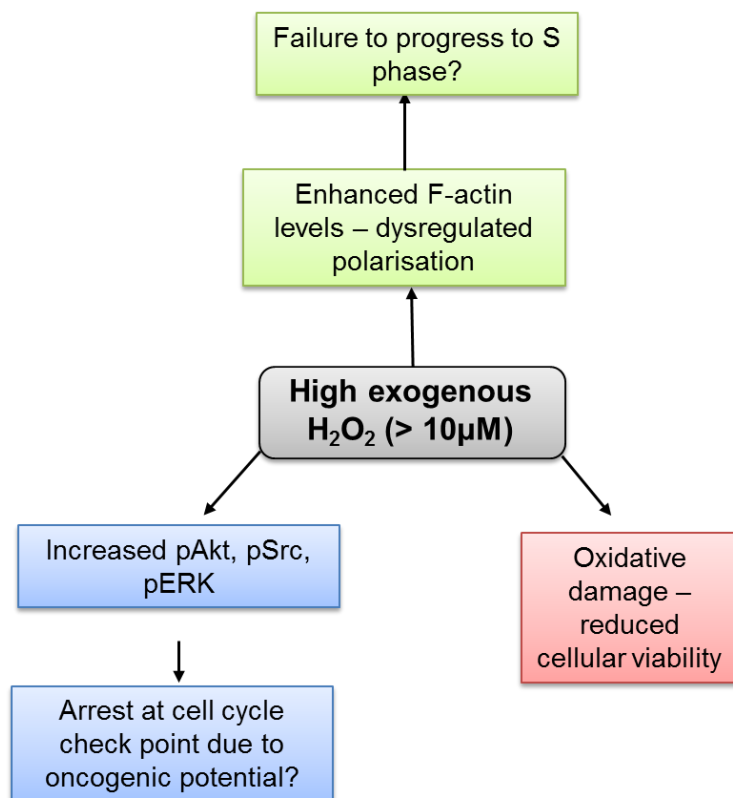


Figure 4.23 Model for high exogenous H_2O_2 causing decreased T lymphocyte proliferation

Extracellular scavenging of H_2O_2 with catalase significantly reduced naïve T lymphocyte proliferation, with the highest concentration of catalase completely attenuating cell division. Excitingly, ROS generated upon TCR ligation appears to be required for cellular proliferation. This is in direct contrast to a recent study which observed that catalase had no effect upon human naïve T lymphocyte proliferation (Belikov *et al.*, 2014). In both experiments, cells were treated with catalase for 30

minutes prior to stimulation with CD3/CD8 antibody immobilised upon on microbeads. The only difference in the experimental design was that I used a naïve T lymphocyte isolation kit, whereas Belikov *et al.*, 2014 used a pan T lymphocyte isolation kit. This could again suggest a differential requirement for ROS between T lymphocytes at different developmental stages.

The NADPH enzyme inhibitor DPI also significantly inhibited T lymphocyte proliferation, corroborating the reduced proliferation observed following scavenging of ROS by catalase. Interestingly, DPI has been previously observed to inhibit cellular proliferation of RAT1 fibroblasts by blocking cell cycle progression to the G2 stage. DPI was observed to induce down-regulation of cyclin B and Thr288 phosphorylated Aurora-A levels and ultimately force mitotic exit (Scaife, 2004).

(b) Adhesion

It was important to address whether H₂O₂ treatment could alter the ability of T lymphocytes to adhere, as the adhesion of lymphocytes to components of the extracellular matrix and other cells is critical for successful cell migration, extravasation and the formation of immunological synapses (Hogg *et al.*, 2011). It has been previously reported that H₂O₂ altered β 2- integrin CD11b/CD18-activation and enhanced neutrophil adhesion (Blouin *et al.*, 1999). Furthermore, H₂O₂ has been described to induce VCAM-1 expression in β -cells through extracellular Ca²⁺ influx (Lee *et al.*, 2008) and enhance leukocyte adhesion to endothelial cells via NFK β dependent gene regulation of VCAM-1 (Lee *et al.*, 2007). Contrastingly, H₂O₂ decreased the adhesion of pluripotent stem cells and down-regulated their expression of adhesion receptors although it is conceivable that stem cells may not be resistant to exposure to ROS as they may not be exposed to high levels of ROS under normal physiological conditions, unlike T lymphocytes. Interestingly, although H₂O₂ has been shown to vastly affect the adhesion process in other immune cells types, it has not been extensively studied upon TCR stimulated T lymphocyte adhesion prior to the work reported here. Surprisingly, H₂O₂ had no effect upon both basal and TCR-stimulated adhesion to fibronectin or on expression the integrin receptors, LFA-1 (CD11a expression) and α 4 β 1 (CD49d expression) in SEB-activated T lymphocytes. In addition, as H₂O₂ had no effect upon basal T lymphocyte adhesion, it is unlikely that a defect in adhesion underlies the suppression in migration observed with H₂O₂

The roles of ROS in chemokine-induced signalling, migration and polarisation

H₂O₂ does not act as an *in vitro* chemoattractant for human T lymphocytes, which was especially apparent when it was compared to other known chemoattractants CXCL11, CXCL10 and CXCL12. This is in contrast to the finding that H₂O₂-induced chemotaxis of mouse peritoneal neutrophils (Klyubin *et al.*, 1996) and human neutrophils (Yoo *et al.*, 2011) and that H₂O₂ is an early danger cue required for leukocyte recruitment to sites of tissue damage in *in vivo* models of inflammation (Niethammer *et al.*, 2009; Moreira *et al.*, 2010) [Figure 1.16]. This could have physiological importance, leukocytes are required early after wounding, however lymphocytes are not required until later in the wound repair process and therefore their migratory cues could be distinct (Figure 5.22). However, it is important to note that trans-well migration assays cannot fully model the role of H₂O₂ *in vivo*. In particular, they fail to model the tissue-scale gradient of H₂O₂ that is generated at a wound site which extends between 100-200µm into the tail fin (Niethammer *et al.*, 2009). Indeed, as the concentration of H₂O₂ was not monitored over the course of the experiment it is not known how rapidly it was degraded/ diffused. Therefore, a sustained gradient with continuous production of H₂O₂ might have a distinct effect upon T lymphocyte migration.

To expand on the interesting observation that H₂O₂ actually reduces basal T lymphocyte migration, the protocol was adapted to determine the effect of treating the cells with H₂O₂ then observing basal and chemokine-stimulated migration. Thus, instead of investigating H₂O₂ as a chemoattractant, this adapted protocol sought to determine the effect of H₂O₂ being present in the microenvironment of the T lymphocyte on its migratory response. ROS levels are enhanced in several pathologies that T lymphocytes play an important role, such as cancer and rheumatoid arthritis (Griffiths, 2005; Kesarwani *et al.*, 2013). Cells were pre-treated with H₂O₂ before examining their migration towards either basal or chemokine containing media. Interestingly, H₂O₂ pre-treatment induced a selective and potent inhibition CXCL11-mediated migration of SEB-activated T-lymphocytes. Inhibition of CXCL11-induced migration was more potent than the inhibition of basal migration. CXCL11-induced migration was also more potently inhibited than CXCL12- and CXCL10-induced SEB-activated T lymphocyte migration and naïve T lymphocyte migration which was only affected by high concentrations of H₂O₂. H₂O₂ appears to discriminate between ligands and inhibits CXCL11 induced-migration more than basal migration. This implies that

H₂O₂ could have a precise signalling effect upon CXCR3 receptor which will be investigated further in Chapter 5.

Consistently with H₂O₂, the SOD dismutase mimetic MnTBAP chloride also inhibited the migration of SEB-activated T lymphocytes, but had no effect upon naïve T lymphocytes. Interestingly, dampening the ROS levels using catalase significantly enhanced both basal, CXCL11-induced SEB-activated and CXCL12-induced naïve T lymphocyte migration. Although CXCL12 is known to stimulate ROS in naïve T lymphocytes (Hara-Chikuma *et al.*, 2012), the fact that basal migration was also enhanced suggests that basally migrating cells are producing and impeded by ROS. It would be very interesting to determine the levels of ROS in basally migrating cells. Cells that are metabolically active with increased energy demands are known to produce higher mitochondrial derived ROS (Leone *et al.* 2011; Kluge *et al.*, 2013), however the level of mitochondrial ROS production during T lymphocyte migration is unknown. Inhibition of NOX enzyme with DPI had no effect upon T lymphocyte migration, which implies the ROS suppressing T lymphocyte migration is not generated by NOX enzymes. Therefore, it was particularly interesting to determine the effect of hypoxia on T lymphocyte migration as there are a number of studies suggesting that under hypoxic conditions mitochondria increase their ROS release from Complex III (Guzy *et al.*, 2005). 5% O₂ reduced SEB-activated T lymphocyte migration, consistent with the theory that mitochondrial ROS suppresses migration, which could imply that mitochondria are a source of ROS that dampen T lymphocyte migration (Table 4.1).

	H ₂ O ₂	MnTBAP chloride (SOD mimetic)	Catalase	DPI (NOX inhibitor)	Hypoxia
Basal migration	↓10μM	↓	↑	-	-
CXCL11-induced migration	↓1nM	↓	↑	-	↓

Table 4.1 Effect of ROS manipulation upon basal and CXCL11 stimulated migration in SEB-activated T lymphocytes. ↓ indicates inhibition of migration, ↑ indicates enhanced migration and – no effect upon cell migration.

H₂O₂ appears to have an important role in actin regulation, as it has been previously shown that preventing H₂O₂ uptake impairs actin polymerisation in murine T lymphocytes (Hara-Chikuma *et al.*, 2012) whilst oxidative stress enhances actin polymerisation and impairs actin polarisation to chemokine stimulation in human naïve T lymphocytes (Klemke *et al.*, 2008). H₂O₂ significantly increased the level of F-actin detected and inhibited actin polarisation to CXCL11 in activated T lymphocytes. The ability of H₂O₂ to alter the polarisation and dysregulate the production of F-actin is likely to be one mechanism underlying the ability of high concentrations of H₂O₂ to inhibit actin mediated processes such as migration and proliferation (Figure 5.22).

Perspectives

ROS potentially inhibiting T lymphocyte migration was unexpected and has unearthed several interesting questions. What are the physiological consequences of low levels of ROS suppressing migration? There are several hypothetical scenarios (1) H₂O₂ could dampen the recruitment of adaptive immune cells which are required later in the wound repair process (2) in contrast, low level ROS could be responsible for capturing T lymphocytes at a site of high ROS by restricting their migration and (3) in a pathological setting, high ROS levels cause wide-spread suppression T lymphocyte migration and aid cancer cell survival or prolong chronic infection (Figure 5.22). In addition, the study by Hara-Chikuma *et al* 2012 indicated that H₂O₂ uptake through aquaporin 3 is required for CXCL12-induced mouse T lymphocyte migration; why the disparity? This could be due to interspecies variation between mouse and human cells, and indicates that further investigation into the role of aquaporin 3 and H₂O₂ should be undertaken in human T lymphocytes.

CHAPTER 5: Investigating H₂O₂-induced signalling in T lymphocytes

5.1 Rationale

As demonstrated in the previous chapter, exogenous H₂O₂ potently inhibits T lymphocyte migration. Unravelling the mechanism through which H₂O₂ modulates signalling pathways is vital for understanding its role in T lymphocyte biology. It is widely accepted in the literature that H₂O₂ and ROS can act as second messengers through their ability to reversibly oxidize specific cysteine residues in proteins (D'Autreaux *et al.*, 2007). Indeed, ROS can oxidise phosphatases (Denu *et al.*, 1998), kinases (Yoo *et al.*, 2011) transcription factors (Haddad, 2002) and ion channels (Bogeski *et al.*, 2010) to alter intracellular signalling. This chapter focuses on the ability of H₂O₂ to alter intracellular kinases and phosphatases.

Lyn has been indicated to act as a redox sensor, which is required for neutrophil recruitment to wounds in zebrafish larvae (Yoo *et al.*, 2011). Lyn is a SFK, known for their widespread cellular functions including cell migration. The critical residue which is oxidised in Lyn is cysteine 466 and importantly, this is conserved between species and SFK members. SFK isoforms which are highly expressed in T lymphocytes are Fyn and Lyn (Cooke *et al.*, 1989; Olszowy *et al.*, 1995). SFK oxidation has also been shown to be important in cell adhesion and cytoskeletal rearrangements in fibroblasts (Giannoni *et al.*, 2005).

In addition, Spleen tyrosine kinase family members (SYK) are also key signalling mediators downstream of SFK involved in a wide array of cellular processes including survival, proliferation and migration. SYK are required for H₂O₂ release in neutrophils (Fernandez *et al.*, 1998) and are essential for oxidative stress mediated Ca²⁺ release in B cells (Takano *et al.*, 2002).

Finally, H₂O₂ has been observed to enhance PI3K signalling through the inactivation of phosphatase PTEN (Lee *et al.*, 2002; Kwon *et al.*, 2004). PTEN is a negative regulator of PI3K signalling which converts PI(3,4,5)P₃ to PI(4,5)P₂, thus augmenting PI3K/Akt signalling. PI3K signalling is known to contribute to several cellular processes including migration. SHIP-1 is also of particular interest as although H₂O₂ inactivates PTEN, its effects upon SHIP-1 have not been determined. SHIP-1 converts PI(3,4,5)P₃ to PI(3,4)P₂ and has been shown to be vital in T lymphocyte migration in Chapter 3.

5.1.1 Aims

This chapter aims to examine the contributions of SFKs, SYKs, PI3K and SHIP-1 in ligand and H₂O₂-mediated signalling in human T lymphocytes

In particular I will:

- examine the contribution of SFKs, SYK and PI3K signalling pathways in basal and CXCL11 stimulated migration.
- determine whether H₂O₂ signals through PI3K, SYK or SFK signalling pathways to manipulate T lymphocyte functions.
- examine both oxidative signalling and oxidative stress responses by using a wide range of H₂O₂ concentrations.

5.2 The importance of PI3K, SFK and SYK signalling in activated T lymphocyte migration

5.2.1 SFK and SYK signalling is required for SEB-activated T lymphocyte migration

The Neuroprobe migration assay was used to identify the contributions of SFK and SYK in SEB-activated T lymphocyte migration. SEB-activated T lymphocytes were pre-treated with increasing concentration of the pan-SFK inhibitor PP2 for 30 minutes prior to being plated in the cell migration assay and exposed to either basal media or CXCL11. PP2 displays considerable selectivity for SFK family members, although it does not discriminate between different members. An *in vitro* assay with human peripheral blood the IC₅₀ values of PP2 against Lck (4nM), Hck (5nM), Fyn (5nM), JAK2 (>50µM) and EGF-R (480nM) (Hanke *et al.*, 1996). In a later, more thorough study PP2 inhibited Src and Lck with IC₅₀ values of 50nM with a 3-10 fold lower potency at Csk, p38a MAPK and CK1d (Bain *et al.*, 2007). In my experiments 1nM PP2 caused a significant suppression of SEB-activated T lymphocyte migration in response to CXCL11, whereas basal migration was significantly suppressed at 100µM (Figure 5.1 A). Thus, SFK signalling is required for directional migration of SEB-activated T lymphocytes and SFKs signalling is important following CXCL11 induced signalling.

Secondly, SEB-activated T lymphocytes were pre-treated with increasing concentrations of the pan SYK-inhibitor piceatannol for 30 minutes. Picetannol (4-[(1E)-2-(3,5-Dihydroxyphenyl)ethenyl]-1,2-benzenediol) is a resverate analogue that exhibits anti-proliferative and anti-inflammatory effects (Geahlen *et al.*, 1989). It inhibits protein tyrosine kinases by competing for the tyrosine-containing substrate binding site. Picetannol inhibits Syk with an IC₅₀ of 10µM and offers 10 fold selectively over inhibition of SFK kinase Lyn (Geahlen *et al.*, 1989). However, picetannol also inhibits other protein tyrosine kinases, with IC₅₀s against Lck of 15µM (Ashikawa *et al.*, 2002). In my experiments, CXCL11 driven migration of SEB-activated lymphocytes was significantly inhibited following incubation with 1µM piceatannol, however basal migration was not affected (Figure 5.1 B). This implies that SYK signalling is required for CXCL11-induced directional but not basal migration of SEB-activated T lymphocytes.

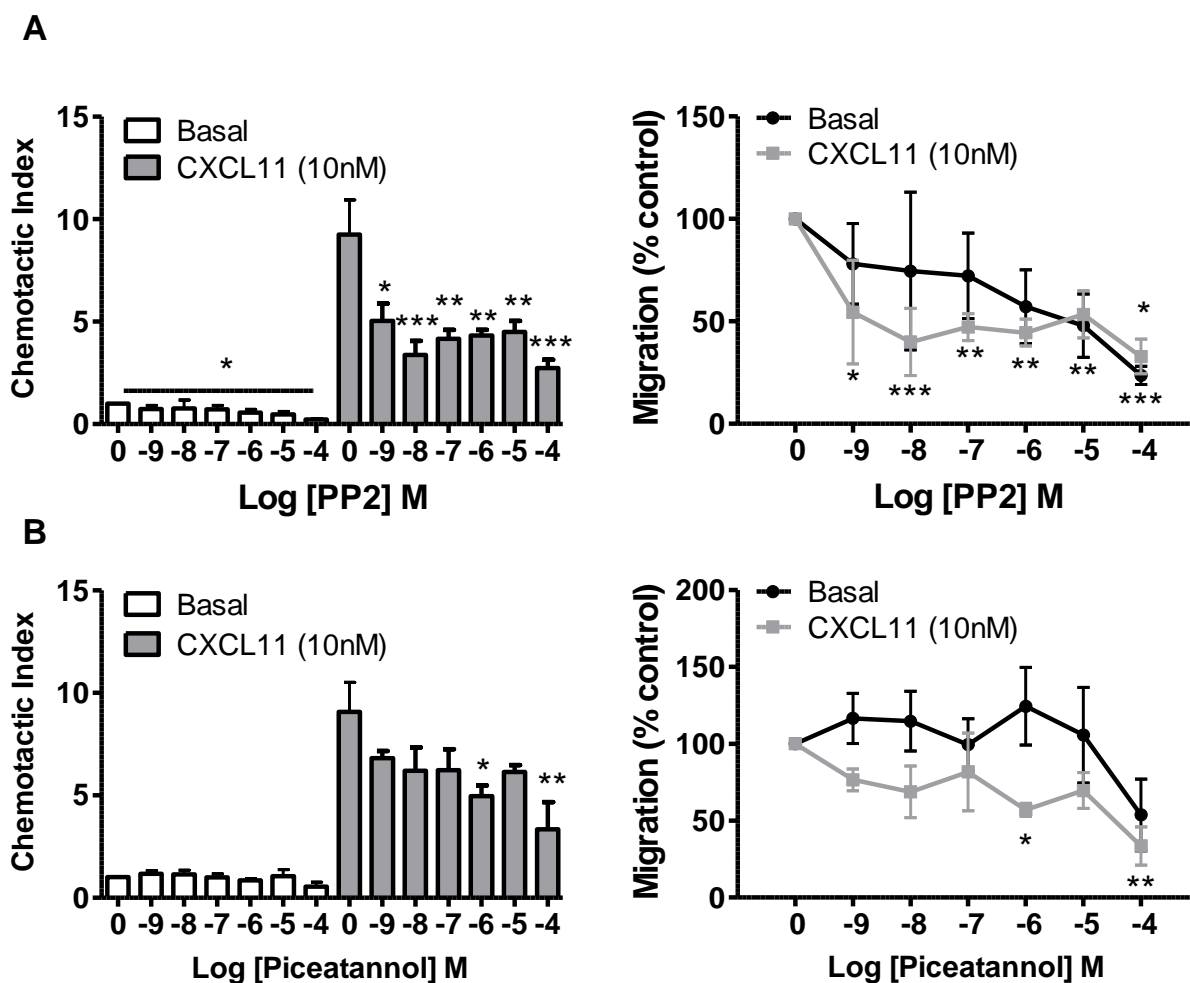


Figure 5.1 Effect of SFK inhibitor PP2 and SYK kinase inhibitor piceatannol upon basal and CXCL11-induced SEB-activated T lymphocyte migration. SEB-activated T lymphocytes were washed in serum free media and resuspended at 3.2 x million cells per ml. Cells were pre-treated with either vehicle or stated concentrations of (A) PP2 or (B) piceatannol for 30 minutes. The *in vitro* basal migration of cells and migration towards the chemokine CXCL11 (10nM) was then assessed by Neuroprobe assay. Data are expressed as chemotactic index (Left panel) or as percentage of control migration (Right panel) of at least three independent donors with samples run in duplicate. Data are the mean \pm SEM whilst statistical significance was determined using a one-way ANOVA with Dunnett's post-test where * p <0.05, ** p <0.01 and *** p <0.001 compared to control.

5.2.2 Simultaneous treatment of SFK inhibitors with exogenous H₂O₂ could not rescue the migratory defect observed with H₂O₂

SFK have also been linked with H₂O₂-induced chemotaxis in zebrafish wounding models (Niethammer *et al.*, 2009). Therefore, a simplistic *in vitro* model was utilised to determine whether SFK and H₂O₂ could signal via the same pathway in human T lymphocytes. The effect of inhibiting SFK alone, exogenous H₂O₂ alone or treating cells with both in combination was compared.

SEB-activated cells were treated with 1µM PP2 alone, 10nM H₂O₂ alone or both 1µM PP2 and 10nM H₂O₂ in combination before being exposed to a chemoattractant in the migration assay. All treatment combinations exhibited a similar inhibition of CXCL11-induced migration, with no significant differences between when a cell was treated with H₂O₂ or PP2 alone compared to treatments given in combination (Figure 5.2 A).

5.2.3 Simultaneous treatment of SYK or PI3K inhibitors with exogenous H₂O₂ could not rescue the migratory defect observed with exogenous H₂O₂ treatment

The same protocol was utilised to establish whether either SYK or PI3K and H₂O₂ could signal through the same pathway in human T lymphocytes. Firstly, SEB-activated cells were treated with 1µM piceatannol alone, 10nM H₂O₂ alone or both 1µM piceatannol and 10nM H₂O₂ in combination before being exposed to a chemoattractant in the migration assay. All treatment combinations exhibited a similar inhibition of CXCL11-induced migration, with no significant differences when a cell was treated with H₂O₂ or piceatannol alone compared to treatments given in combination (Figure 5.2 B).

Secondly, SEB-activated cells were treated with 10µM ZSTK474 alone, 10nM H₂O₂ alone or both 10µM ZSTK474 and 10nM H₂O₂ in combination before being exposed to a chemoattractant in the migration assay. 10µM ZSTK474 alone caused a very pronounced inhibition of basal and chemokine-induced SEB-activated T cell migration (Figure 5.2 C). This was an interesting observation as it has been previously observed that pan PI3K inhibition with LY294002 has no effect upon directional migration of SEB-activated T lymphocytes (Smit *et al.*, 2003; Cronshaw *et al.*, 2006). Here, 10µM ZSTK474 significantly impaired basal migration to 37.1 ± 10.2 % and CXCL11-driven migration to 4.7 ± 1.5 % of control. However there was no significant difference between ZSTK474 treatment alone and ZSTK474 plus H₂O₂ upon migration (Figure 5.2 C).

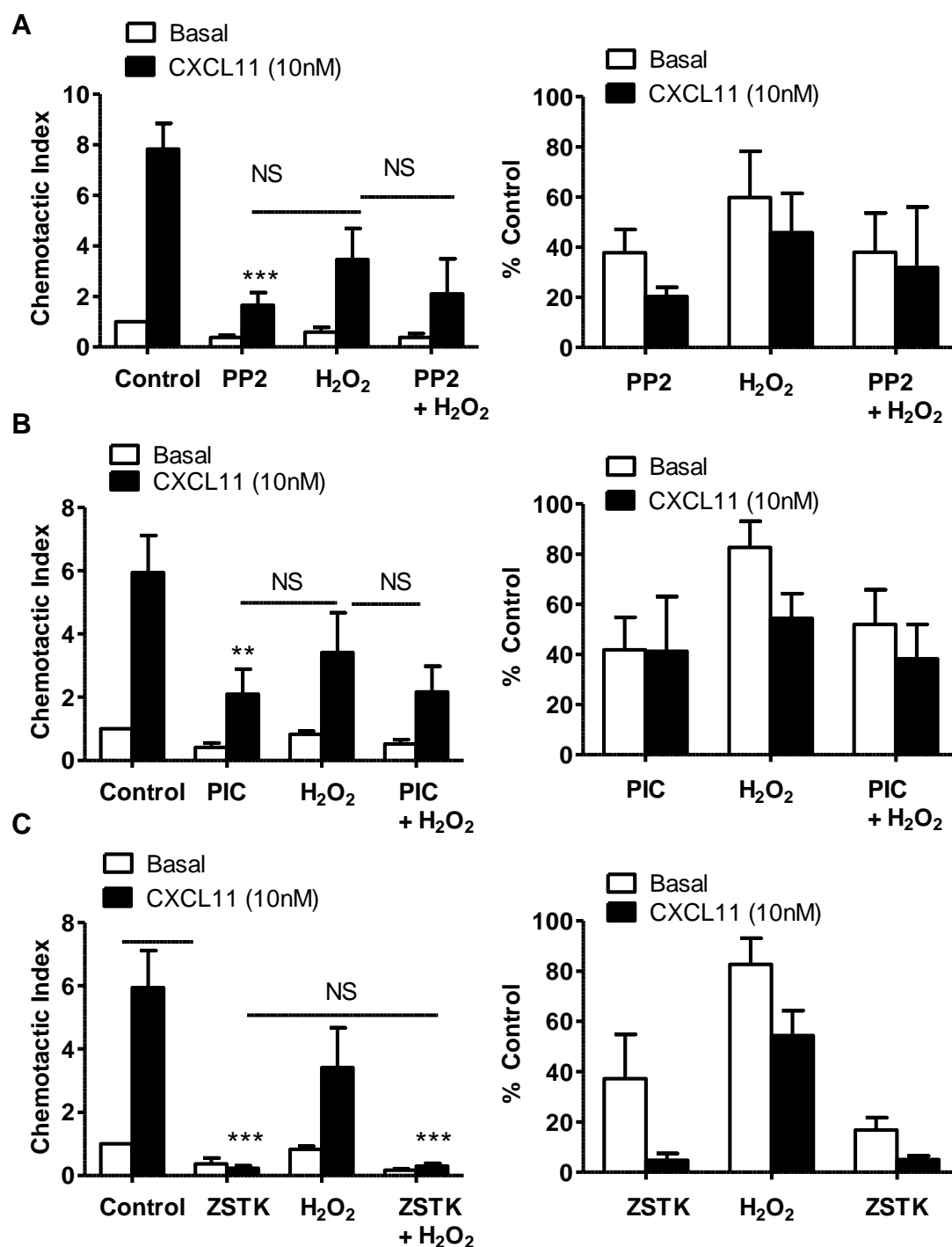


Figure 5.2 SFK, SYK or PI3K inhibition does not rescue or exacerbate the migratory defect observed with H₂O₂. SEB-activated T lymphocytes were washed in serum free media and resuspended at 3.2 million cells per ml and pre-treated with (A) 1 μ M PP2 alone, 10nM H₂O₂ alone or PP2 and H₂O₂ in combination (B) 1 μ M picetannol (PIC) alone, 10nM H₂O₂ alone or PIC and H₂O₂ in combination and (C) 10 μ M ZSTK474 alone, 10nM H₂O₂ alone or ZSTK474 and H₂O₂ in combination. The *in vitro* basal migration of cells and migration towards the chemokine CXCL11 (10nM) was then assessed by Neuroprobe assay. Data are expressed as chemotactic index (Left panel) or as percentage of control migration (Right panel) of at least three independent donors with samples run in duplicate. Data are the mean \pm SEM and statistical testing using a two-way ANOVA with a Bonferroni post-test whereby significance is represented by ** $p < 0.01$ *** $p < 0.001$ compared to control.

5.2.4 PI3K signalling is required for basal and CXCL11-induced migration of SEB-activated T lymphocyte

It has previously been shown that chemokine-induced migration of activated T lymphocytes is resistant to pan PI3K inhibitors LY294002 and wortmannin (Smit *et al.*, 2003; Cronshaw *et al.*, 2006; Smith *et al.*, 2007). However the pan PI3K inhibitor, ZSTK474 inhibited both basal and CXCL11 stimulated migration in Figure 5.2 C. Therefore, it was interesting to compare the effect of different PI3K inhibitors on the directed migration of SEB-activated T lymphocytes. The IC₅₀ of each inhibitor at each PI3K Class IA isoform are described in Table 1.3.

First, SEB-activated T lymphocytes were pre-treated with increasing concentration of LY294002 for 30 minutes prior to being plated in the cell migration assay and exposed to either basal media or CXCL11. LY294002 inhibited basal migration but had no effect upon directional migration (Figure 5.3 A) consistent with previous findings (Smith *et al.*, 2007). Next, cells were treated with ZSTK474, which significantly impaired basal to 16.16 ± 6.67 % and CXCL11-induced migration to 41.28 ± 12.80 % of control (Figure 5.3 B)

As migration was inhibited by a pan PI3K inhibitor (ZSTK474) the effect of isoform specific PI3K inhibitors was examined. Neither PI3K γ inhibitor AS-605240 (Figure 5.4 A) nor PI3K δ inhibitor IC871114 (Figure 5.4 B) had any effect upon basal or CXCL11 migration. However, as different isoforms of PI3K have redundant roles, the effect combining AS-605240 and IC871114 was examined. The combination of both inhibitors significantly impaired both basal to 28.48 ± 18.97 % and CXCL11-induced migration to 53.84 ± 15.93 % of control, in a similar fashion to ZSTK474 (Figure 5.4 C). Therefore it appears like PI3K γ and δ isoform signalling is required for CXCL11-induced migration of SEB-activated T lymphocytes though there is a residual migratory response resistant to inhibitors of these isoforms

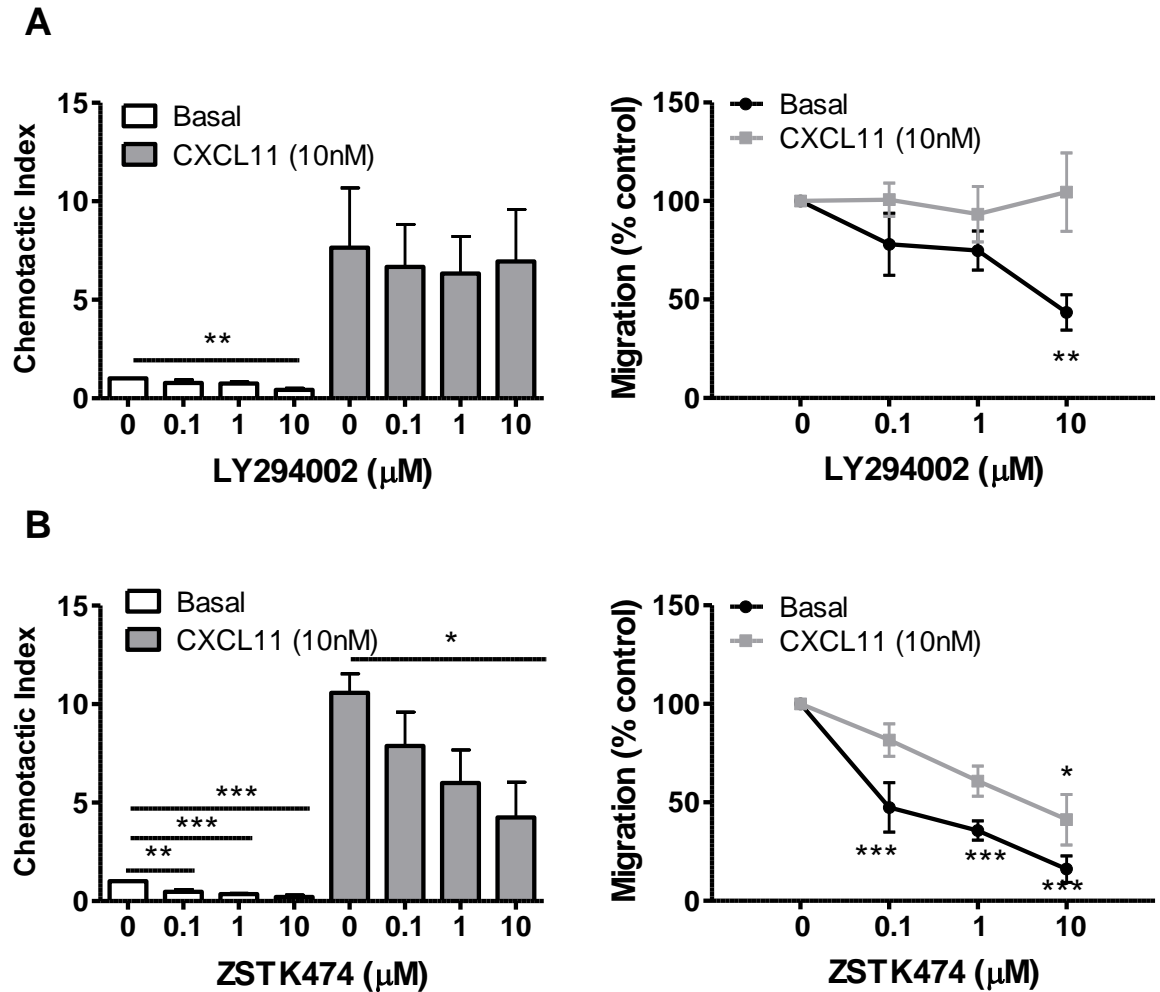


Figure 5.3 Pan PI3K inhibition by ZSTK474 and LY294002 inhibit basal and CXCL11-induced migration of SEB-activated T lymphocyte. SEB-activated T lymphocytes were washed in serum free media and resuspended at 3.2 million cells per ml. Cells were pre-treated with increasing concentrations of (A) LY294002 or (B) ZSTK474 for 30 minutes. The basal migration and migration towards the chemokine CXCL11 (10nM) was then assessed by the Neuroprobe assay. Data are expressed as chemotactic index (Left panel) or as percentage of control migration (Right panel). Data are the mean \pm SEM of three independent donors. Statistical significance was determined using a one-way ANOVA with Dunnett's post-test where * p <0.05, ** p <0.01 and *** p <0.001 compared to control.

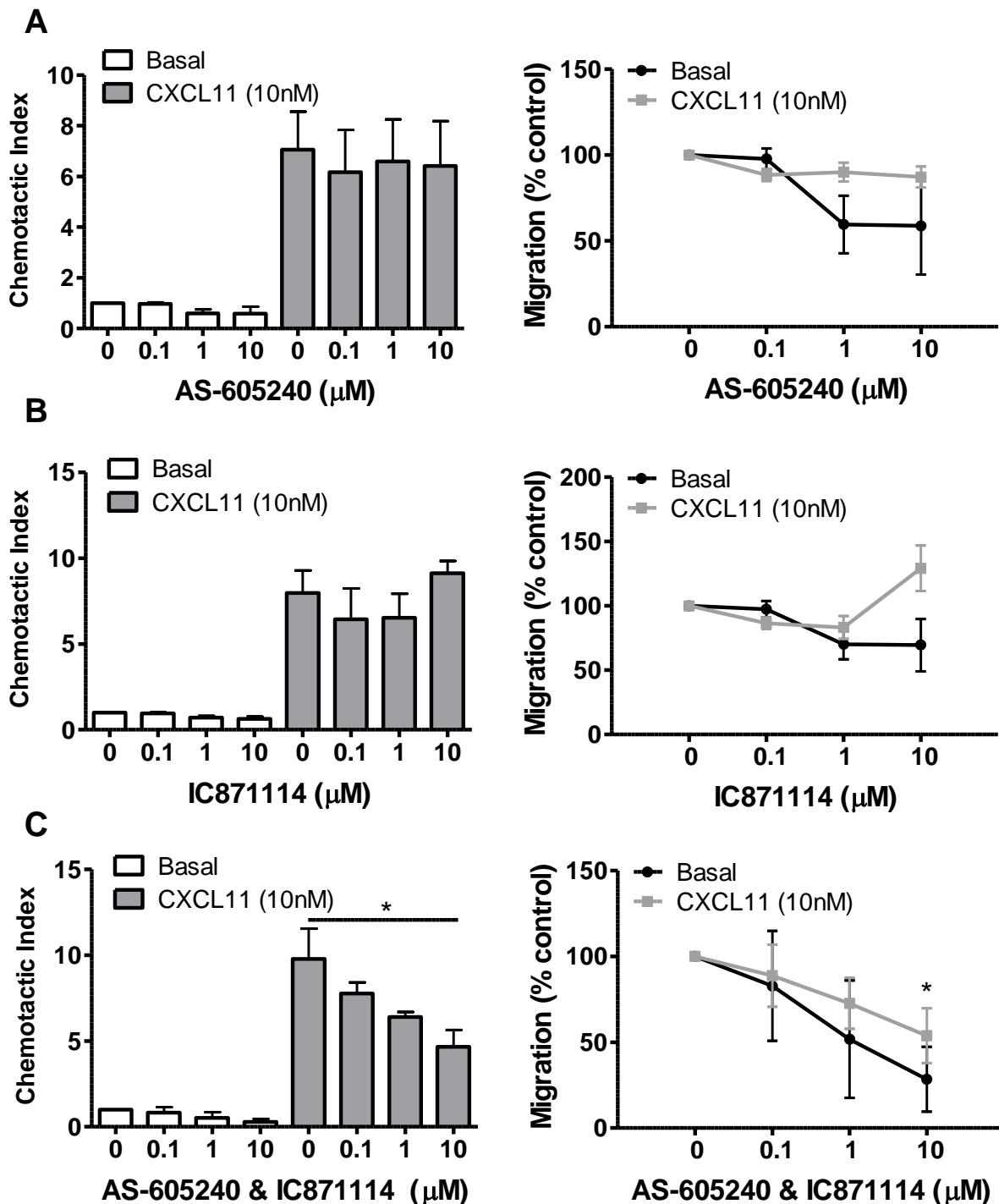


Figure 5.4 Basal and CXCL11-induced SEB-activated T lymphocyte migration is not abrogated by specific inhibition of PI3K δ and PI3K γ . SEB-activated T lymphocytes were washed in serum free media and resuspended at 3.2 million cells per ml. Cells were pre-treated with either vehicle or stated concentrations of (A) IC871114, (B) AS-605240 or (C) IC871114 & AS-605240 for 30 minutes. The basal migration of cells and migration towards the chemokine CXCL11 (10nM) was then assessed by the Neuroprobe assay. Data are expressed as chemotactic index (Left panel) or as percentage of control migration (Right panel). Data are the mean \pm SEM of three independent donors. Statistical significance was determined using a one-way ANOVA with Dunnett's post-test where * $p < 0.05$ compared to control.

5.2.5 PLC is required for SEB-activated T lymphocyte migration to CXCL11

Although PI3K appears to be non-dispensable for SEB-activated T lymphocyte migration, inhibition did not completely abrogate migration, which could suggest that SEB-activated T lymphocyte migration may also require signalling through PI3K-independent pathways. PLC is required for PI[4,5]P₂ hydrolysis into two second messengers diacylglycerol (DAG) and inositol triphosphate (IP[1,4,5]P₃) and IP₃ binds to the IP₃ receptor (IP₃R), and evokes the release of Ca²⁺ from intracellular endoplasmic reticulum (ER) Ca²⁺ stores in a PI3K independent manner. CXCL11 has been shown to elicit robust elevation of Ca²⁺ (Figure 3.10). DAG binds to PKC to initiate signalling, and has also been found to be involved in migration of T cells (Cronshaw *et al.*, 2006).

Therefore the roles of PLC and PKC in the directional migration of human SEB-activated T were investigated. SEB activated T lymphocytes were pre-treated with 1µM PLC inhibitor U73122 (1-[6-[[[(17β)-3-Methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione), an inactive analog of U73122 (U73343) or the PKC inhibitor RO-32-0432 for 30 minutes prior to being plated in the Neuroprobe migration assay and exposed to either basal media or 10nM CXCL11. PLC inhibitor U73122 inhibits IP₃-mediated Ca²⁺ release at concentrations of 1–2 µM, however it does not reduce the production of IP₃, used as an indicator of PLC activity, until higher concentration of 10µM and above (Smallridge *et al.*, 1992; Alter *et al.*, 1994; Hellberg *et al.*, 1996). Ro-32-0432 is a cell-permeable selective inhibitor of PKC which according to Santa Cruz Biotechnology displays a 10-fold greater selectivity for PKCα (IC₅₀ = 9.3 nM); and a 4-fold greater selectivity for PKC-β over PKCε (IC₅₀ = 108 nM).

U73122 significantly inhibited both basal and CXCL11-induced migration consistent with previous findings (Cronshaw *et al.*, 2006). The inactive analog, U73343 did exhibit a smaller but significant decrease in basal migration but had no effect upon CXCL11-induced migration. Finally, PKC inhibition significantly decreased basal but had no effect upon CXCL11-induced migration (Figure 5.5). This implies that PLC induced signalling is vital for SEB-activated lymphocyte basal and directional migration, however PKC signalling through DAG is only important in basal migration.

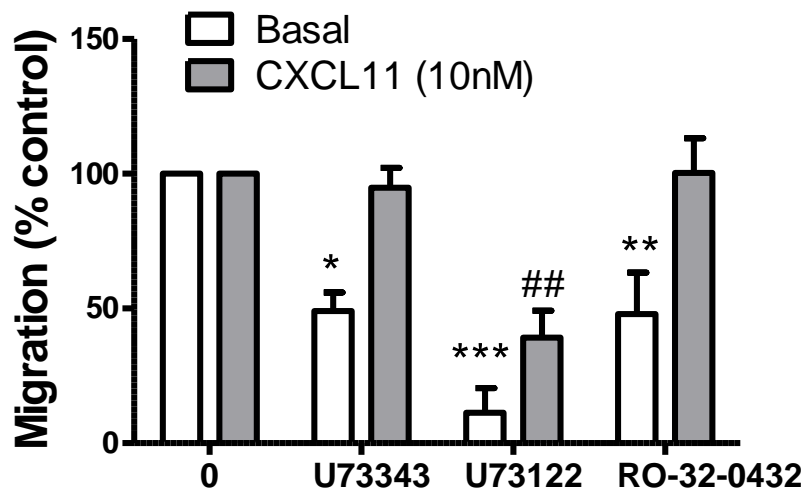


Figure 5.5 PLC, but not PKC, is required for SEB-activated T lymphocyte migration towards CXCL11. SEB-activated T lymphocytes were washed in serum free media and resuspended at 3.2 million cells per ml. Cells were pre-treated with either DMSO vehicle or 1 μ M U73343, U73122 or RO-32-0432 for 30 minutes. The basal migration of cells and migration towards the chemokine CXCL11 (10nM) was then assessed by the Neuroprobe assay. Data are expressed as percentage of control migration. Data are the mean \pm SEM of three independent donors. Statistical significance was determined using a one-way ANOVA with Dunnett's post-test * p <0.05, ** p <0.01 and *** p <0.001 compared to basal and ## p <0.01 compared with CXCL11 control.

5.3 H₂O₂ induces the phosphorylation of Akt, Src and ERK in SEB-activated T lymphocytes

To explore the ability of H₂O₂ to initiate cellular signalling, its ability to activate PI3K, SFK or MAPK kinases in SEB-activated T lymphocytes was examined. Both PI3K and MAPK signalling have been previously implicated as being activated by H₂O₂ (Kefaloyianni *et al.*, 2006; Sadidi *et al.*, 2009; Ha *et al.*, 2012) whilst the SFK member Lyn was determined to have a critical cysteine, cys-466, which is required for it to exhibit redox sensitivity in zebrafish (Yoo *et al.*, 2011).

Akt (Ser473) phosphorylation, c-Src phosphorylation (Tyr416) and ERK1 (Tyr202) and ERK2 (Tyr204) were determined as an indirect measure of PI3K, SFK and MAPK activity, respectively. SEB-activated T lymphocytes were pre-treated with increasing concentrations of H₂O₂ for 30 minutes and then levels of phosphorylated Akt, ERK and Src were determined by Western Blot. H₂O₂ induced a significant increase in Akt phosphorylation at concentrations above 100µM (Figure 5.6 A). Src phosphorylation was significantly increased at 10mM H₂O₂ (Figure 5.6 B). Consistently, ERK phosphorylation was only significantly increased at 10mM H₂O₂ (Figure 5.6 C). H₂O₂ can thus induce PI3K, Src kinase and MAPK signalling but only at high concentrations, which are likely to place cells under oxidative stress and were previously shown to effect cell viability following 3 hour exposure (Figure 4.2).

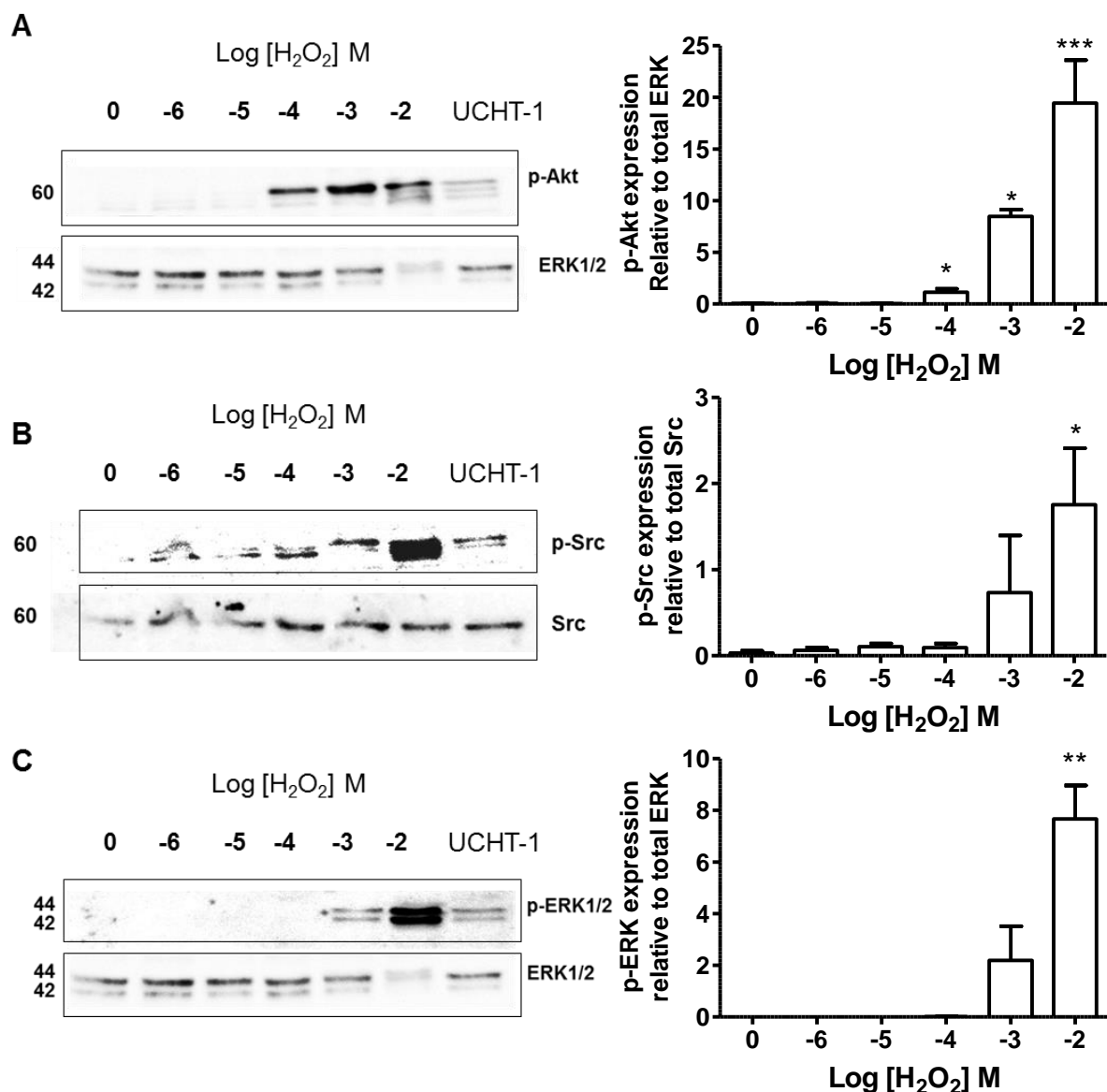


Figure 5.6 High concentrations of H₂O₂ induce the phosphorylation of Akt, ERK and Src kinase in SEB-activated T lymphocytes. SEB-activated T lymphocytes were treated with increasing concentration of H₂O₂ for 30 minutes. Cells were lysed as described, loaded onto poly-acrylamide gels and proteins separated under reducing conditions using SDS-PAGE. Proteins were transferred to nitro-cellulose membrane, blocked and Immunoblotted for (A) p-Akt, (B) p-Src and (C) p-ERK and then stripped and re-probed for ERK1/2. Anti-CD3 antibody (UCHT-1) was used as a positive control. Representative Immunoblots with the units representing kDa (left panel) and the mean \pm SEM of image J quantified blots from three independent donors (right panel) are shown. Statistical significance was determined by one-way ANOVA with Dunnett's post-test whereby significance is represented by * $p < 0.01$, ** $p < 0.05$ or *** $p < 0.001$ compared to control.

5.3.1 PI3K signalling is required for H₂O₂ to induce phosphorylation of Akt

To further verify that H₂O₂ activates PI3K signalling, the effect of pan PI3K inhibitor ZSTK474 on H₂O₂-induced Akt phosphorylation was determined. SEB-activated T lymphocytes were pre-treated with ZSTK474 (1µM) for 30 minutes and then stimulated with CXCL11 (10nM) for 5 minutes to initiate down-stream signalling including PI3K. ZSTK474 completely abolished CXCL11-induced Akt phosphorylation (Figure 5.7 A) verifying that ZSTK474 was indeed functional and able to inhibit PI3K dependent signalling. Cells were stimulated with H₂O₂ (100µM) for 30 minutes and levels of Akt phosphorylation determined by Western Blotting. ZSTK474 pre-treatment significantly reduced the ability of H₂O₂ to induce the phosphorylation of Akt (Figure 5.7 B). Thus, H₂O₂-induced Akt phosphorylation appears to be dependent on PI3K activation.

5.3.2 Catalase pre-treatment completely attenuates H₂O₂-induced phosphorylation of Akt.

The effect of catalase upon Akt phosphorylation was examined and its ability to dampen H₂O₂ signalling verified. SEB-activated T lymphocytes were pre-treated with increasing concentrations of catalase for 30 minutes prior to exposing them to H₂O₂ (100µM) and Akt phosphorylation measured using Western Blotting. Catalase had no effect upon basal Akt phosphorylation and significantly decreased H₂O₂-induced Akt phosphorylation (Figure 5.8 A). Heat-inactivated catalase had no effect upon H₂O₂-induced Akt phosphorylation (Figure 5.8 B).

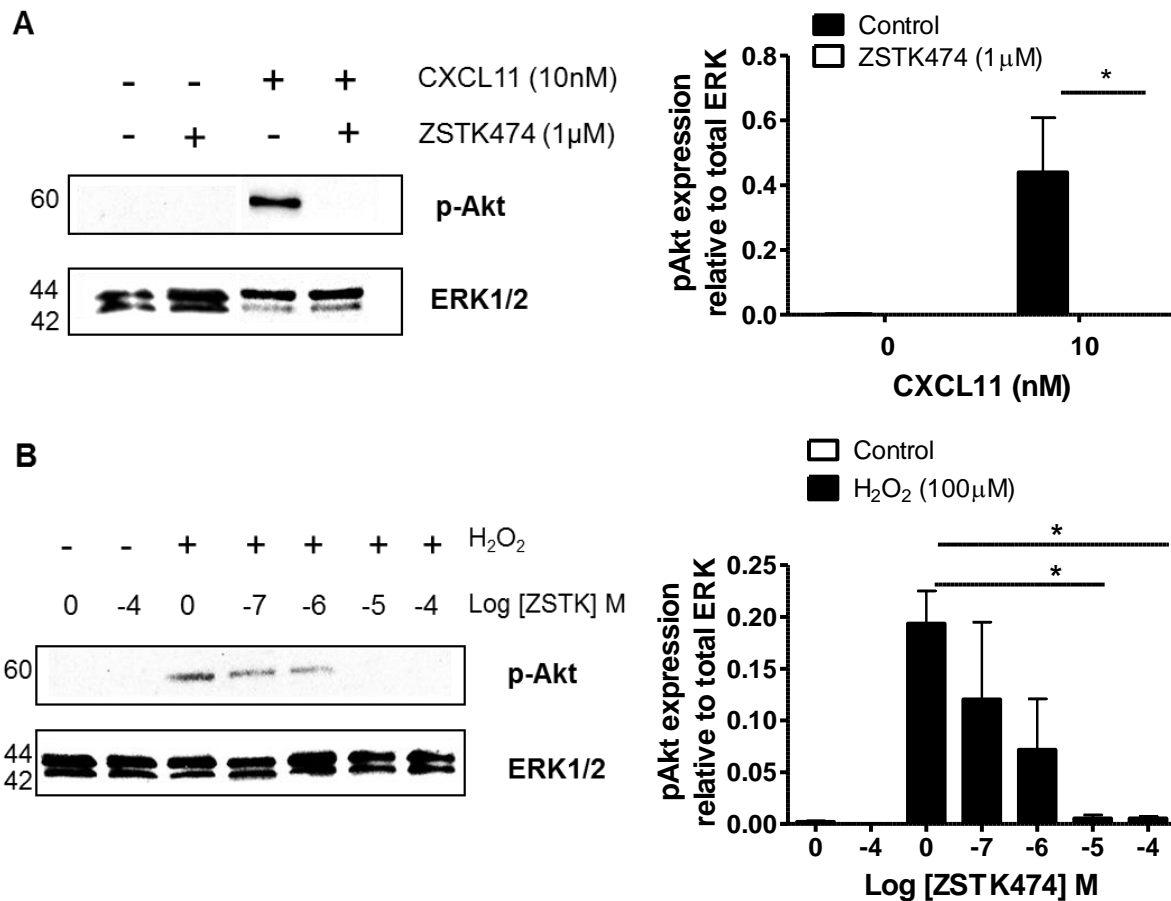


Figure 5.7 PI3K signalling is required for H₂O₂ to induce phosphorylation of Akt in SEB-activated T lymphocytes. SEB-activated T lymphocytes were pre-treated with increasing concentrations of ZSTK474 for 30 minutes. Cells were then treated with (A) 10nM CXCL11 or (B) 100μM H₂O₂ for 30 minutes, lysed, loaded onto poly-acrylamide gels and proteins separated under reducing conditions using SDS-PAGE. Proteins were transferred to nitro-cellulose membrane, blocked and Immunoblotted for p-Akt and then stripped and re-probed for ERK1/2. Representative Immunoblots (left panel) and the mean \pm SEM of image J quantified blots of three independent donors (right panel) are shown. Statistical significance was determined using a (A) two-way ANOVA with Bonferri's post-test and (B) one-way ANOVA with Dunnett's post-test where * p <0.05 compared to control.

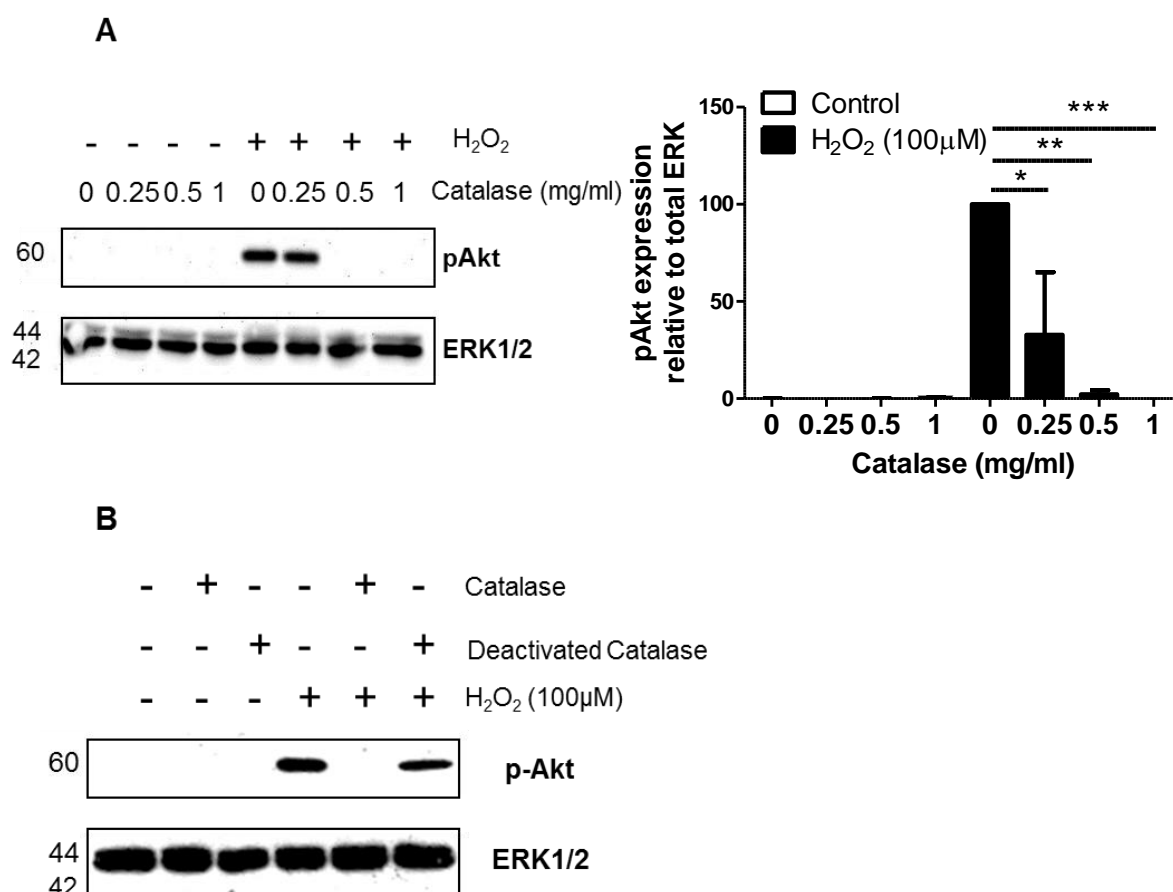


Figure 5.8 Catalase pre-treatment attenuates H₂O₂ induced Akt phosphorylation in SEB-activated T lymphocytes. SEB-activated T lymphocytes were pre-treated with either vehicle control or increasing concentrations of catalase for 30 minutes. Cells were then treated with 100μM H₂O₂ for 30 minutes. Cells were lysed as described, loaded onto poly-acrylamide gels and proteins separated under reducing conditions using SDS-PAGE. Proteins were transferred to nitro-cellulose membrane, blocked and Immunoblotted for p-Akt and then stripped and re-probed for ERK1/2. (A) Data shown are representative Immunoblots, units represent kDa (left panel) and the mean ± SEM of three independent donors of image J quantified blots (right panel). (B) is a representative blot of 2 donors. Statistical significance was determined by a one-way ANOVA with Dunnett's post-test where *p<0.05, **p<0.01 or ***p<0.001 compared to control.

5.4 H₂O₂ enhances the phosphorylation of SHIP-1

SHIP-1 has been shown to enhance cell survival in response to oxidative stress (Gloire *et al.*, 2006). In Chapter 3, SHIP-1 activation negatively impaired T lymphocyte polymerisation, migration and polarisation. Likewise, H₂O₂ was also shown in Chapter 4 to negatively regulate these processes. The effect of exogenous H₂O₂ on SHIP-1 activity was therefore determined. Upon cell stimulation, SHIP-1 is recruited to the membrane where it is tyrosine phosphorylated upon Y¹⁰²⁰, which lies in the second NPXY motif towards its C-terminal. Phosphorylation is used as a marker of activated SHIP-1, when SHIP-1 is located at the cell membrane (Phee *et al.*, 2000) and is able to bind the phosphotyrosine binding (PTB) domains of Shc (Lioubin *et al.*, 1996) plus Dok1 and Dok2 (Dong *et al.*, 2006). SEB-activated T lymphocytes were treated increasing concentrations of H₂O₂ for 30 minutes. Cells were then fixed, permeabilised and level of phosphorylated SHIP-1 assessed using flow cytometry. Increasing concentrations of H₂O₂ increased SHIP-1 phosphorylation in a bell-shaped fashion; with maximal phosphorylation at 1µM (Figure 5.9) SHIP-1 phosphorylation occurs at concentrations of H₂O₂ which do not affect cellular viability but do significantly alter the migration of T lymphocytes. This is an interesting finding that indicates regulation of SHIP-1 phosphorylation could be a mechanism by which H₂O₂ influences T lymphocyte migration.

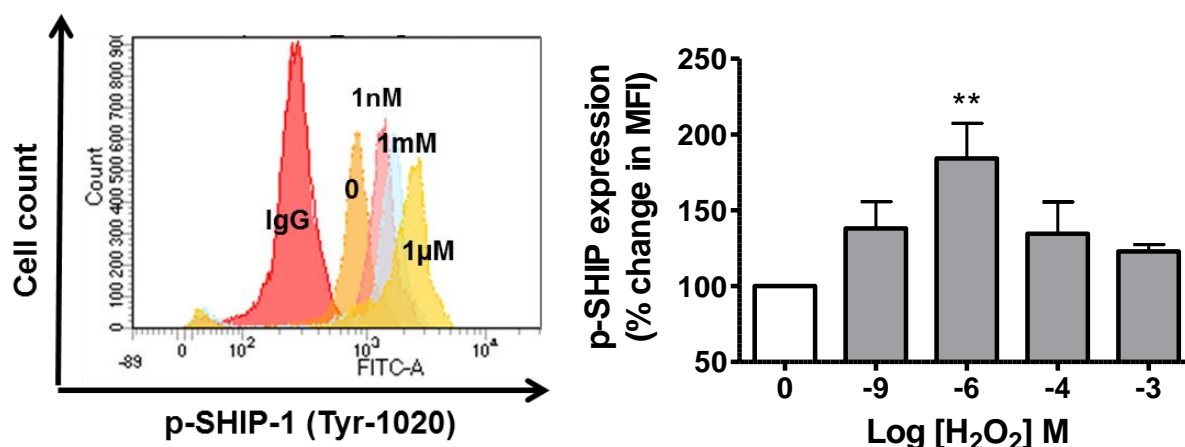


Figure 5.9 H_2O_2 -induced SHIP-1 phosphorylation in SEB-activated T lymphocytes. SEB-activated T lymphocytes were washed three times in serum free media and treated with increasing concentrations of H_2O_2 for 30 minutes. Cells were fixed using BD fixation reagent, permeabilized, incubated with SHIP-1 antibody for 30 minutes followed by FITC conjugated secondary antibody. Mean fluorescence intensity per 10,000 cell was measured using flow cytometry. The left panel is a representative FAC plot and the right panel is the MFI mean \pm SEM of three independent donors expressed as percentage change from control. Statistical significance was determined by one way ANOVA with Dunnett's post-test where ** $p < 0.01$ as compared to control.

5.4.1 H₂O₂ requires SFK signalling to induce phosphorylation of SHIP-1

In B lymphocytes, the phosphorylation of SHIP-1 in response to FcγRIIb stimulation has been observed to be dependent upon Lyn, a SFK member (O'Neill *et al.*, 2011). Although Lyn kinase is not expressed in T lymphocytes (Korade-Mirnic *et al.*, 2000; Scapini *et al.*, 2009), other members of the SFK might be involved in SHIP-1 phosphorylation and therefore the effect of inhibiting SFK upon H₂O₂-induced SHIP-1 phosphorylation was examined.

Firstly, SEB-activated T lymphocytes were pre-treated with 1μM PP2, a non-selective SFK inhibitor, for 30 minutes. Cells were then exposed to increasing concentrations of H₂O₂ and SHIP-1 phosphorylation observed by flow cytometry. Pre-treatment with PP2 significantly impaired H₂O₂-induced SHIP-1 phosphorylation (Figure 5.10 A), indicating that H₂O₂-induced SHIP-1 phosphorylation requires SFK activation.

Lck is a SFK member known to be expressed in T lymphocytes. The role of Lck in SHIP-1 phosphorylation was therefore examined. 1188890-30-3 is an ATP competitive inhibitor of Lck, which according to Calbiochem has an IC₅₀ of 867nM. SEB-activated T lymphocytes were pre-treated with 1μM 1188890-30-3 for 30 minutes, exposed to increasing concentrations H₂O₂ and the level of SHIP-1 phosphorylation determined by flow cytometry. Although 1188890-30-3 completely attenuated SHIP-1 phosphorylation, the basal levels of SHIP-1 phosphorylation was variable between donors. This resulted in the Lck pre-treated SHIP-1 phosphorylation not being significantly different to control (Figure 5.10 B).

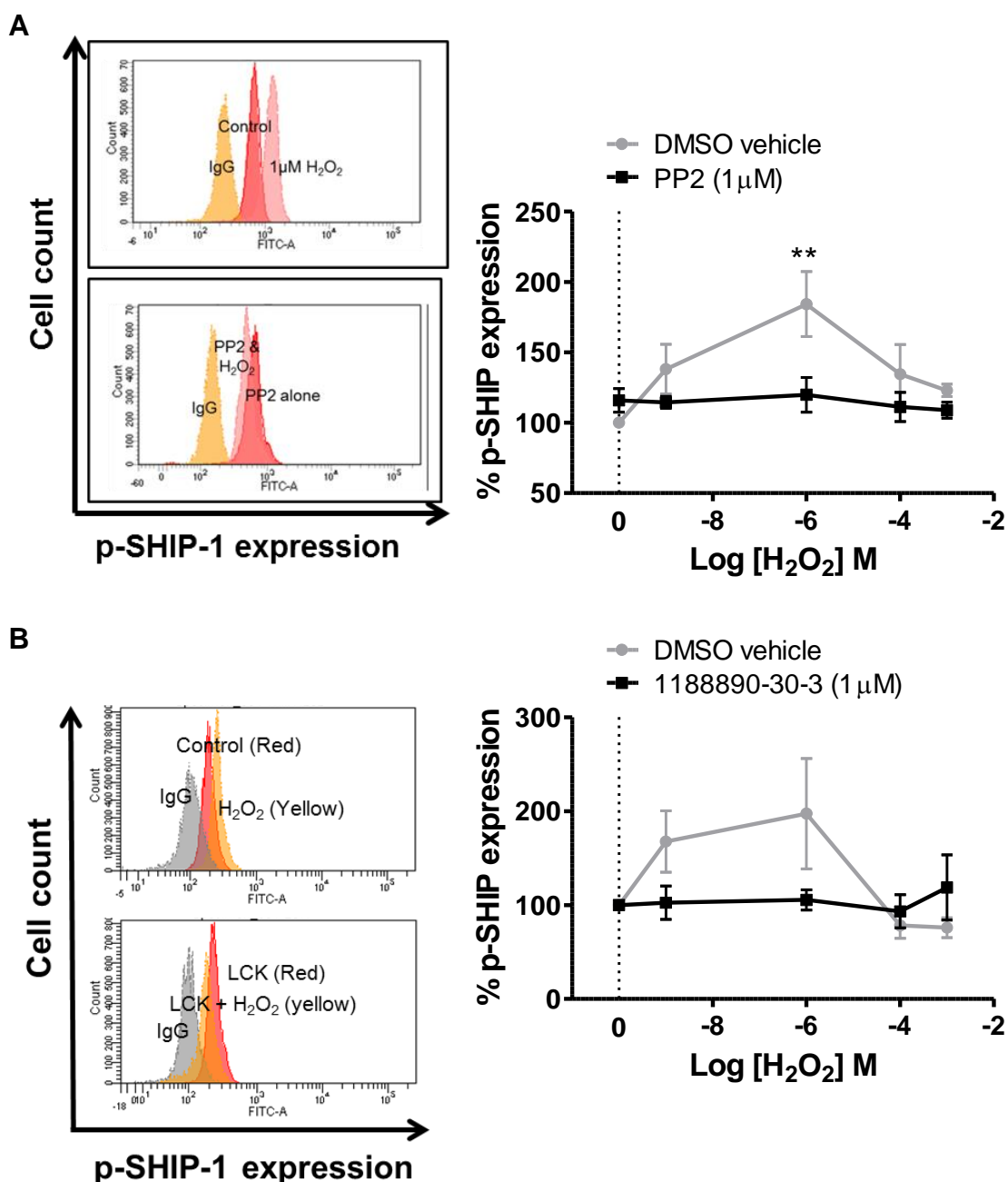


Figure 5.10 Effect of SFK inhibitor (PP2) and Lck inhibitor (1188890-30-3) upon H₂O₂-induced SHIP-1 phosphorylation in SEB-activated T lymphocytes. SEB-activated T lymphocytes were washed three times in serum free media and pre-treated with (A) SFK inhibitor (PP2) and (B) Lck inhibitor (1188890-30-3) for 30 minutes. Cells were fixed using BD fixation reagent, permeabilized, incubated with SHIP-1 antibody for 30 minutes followed by FITC conjugated secondary antibody. Left panel is a representative FAC plot from a single donor and the right panel is the MFI mean \pm SEM of three independent donors expressed as percentage change from control. Statistical significance was determined by one way-ANOVA with Dunnett's post-test where ** $p < 0.01$ as compared to control.

5.4.2 H₂O₂ signalling enhanced the catalytic activity of SHIP-1

Although phosphorylation of SHIP increases recruitment to the plasma membrane and allows for increased SHIP-1 proximity to its substrate, it must be noted that tyrosine phosphorylation has been reported to not directly affect catalytic activity (Phee *et al.*, 2000). The effect of H₂O₂ on the catalytic activity of SHIP-1 was examined. The malachite green assay, which is previously described in section 3.2, was utilised. Briefly, the phosphate product generated by SHIP-1 can be measured using malachite green molybdate; a chemical that reacts with free organic phosphate to form molybdophosphoric acid, which absorbs light at 650nm.

Firstly, the effect of directly treating recombinant SHIP-1 with H₂O₂ was examined. 1µM recombinant SHIP-1 was incubated with increasing concentrations of H₂O₂ for 30 minutes before incubation with *in vitro* substrate inositol 1,3,4,5-tetrakisphosphate (IP₄) for a further 30 minutes. Malachite green molybdate was added to determine the level of free phosphate. H₂O₂ had no effect upon the ability of recombinant SHIP-1 ability to de-phosphorylate IP₄ (Figure 5.11 A). This suggests the H₂O₂ has no direct effect upon recombinant SHIP-1's catalytic activity.

A common problem with recombinant proteins is that they can be misfolded. To combat the possibility of misfolding altering the oxidative sensitivity of the SHIP-1, it was instead immunoprecipitated from SEB-activated T lymphocytes. Immunoprecipitated SHIP-1 was pipetted from one stock aliquot to ensure the level of SHIP-1 protein is equal in each treatment condition. Immunoprecipitated SHIP-1 was treated with 1µM H₂O₂ for 30 minutes before incubation with substrate IP₄. Free phosphate production was determined by addition of malachite green molybdate and absorbance read on a plate reader. Control absorbance from immunoprecipitated SHIP-1 alone was significantly enhanced after incubation with the IP₄ substrate indicating immunoprecipitated SHIP-1 acted as a phosphatase. However H₂O₂ treatment had no direct effect upon immunoprecipitated SHIP-1 catalytic activity (Figure 5.11 B).

The observation that H₂O₂ requires an SFK to phosphorylate SHIP-1 (Figure 5.10 A) suggests that H₂O₂ does not directly oxidise SHIP-1 but instead requires intracellular signalling. As such, it could be hypothesised that the catalytic activity of SHIP-1 could be higher in whole cells treated with H₂O than un-treated cells. Thus, SEB-activated T lymphocytes were treated with media control, 1 or 10µM H₂O₂ for 30 minutes and then SHIP-1 was immunoprecipitated. Half the immunoprecipitated SHIP-1 was plated in a 96 well plate for the malachite green assay and the other half used to verify that the amount of immunoprecipitated SHIP-1 was equal in each group by Western Blot. The

immunoprecipitated SHIP-1 was then incubated with substrate IP₄ and the level of phosphate production determined by the addition of malachite green molybdate. Treatment of the whole cells with 1μM H₂O₂ evoked a significant increase in the catalytic activity of SHIP-1 (Figure 5.11 C). This implies that H₂O₂ induces intracellular signalling to enhance the catalytic activity of SHIP-1. Enhanced SHIP-1 activity within cells could underlie the migratory deficiency observed with H₂O₂

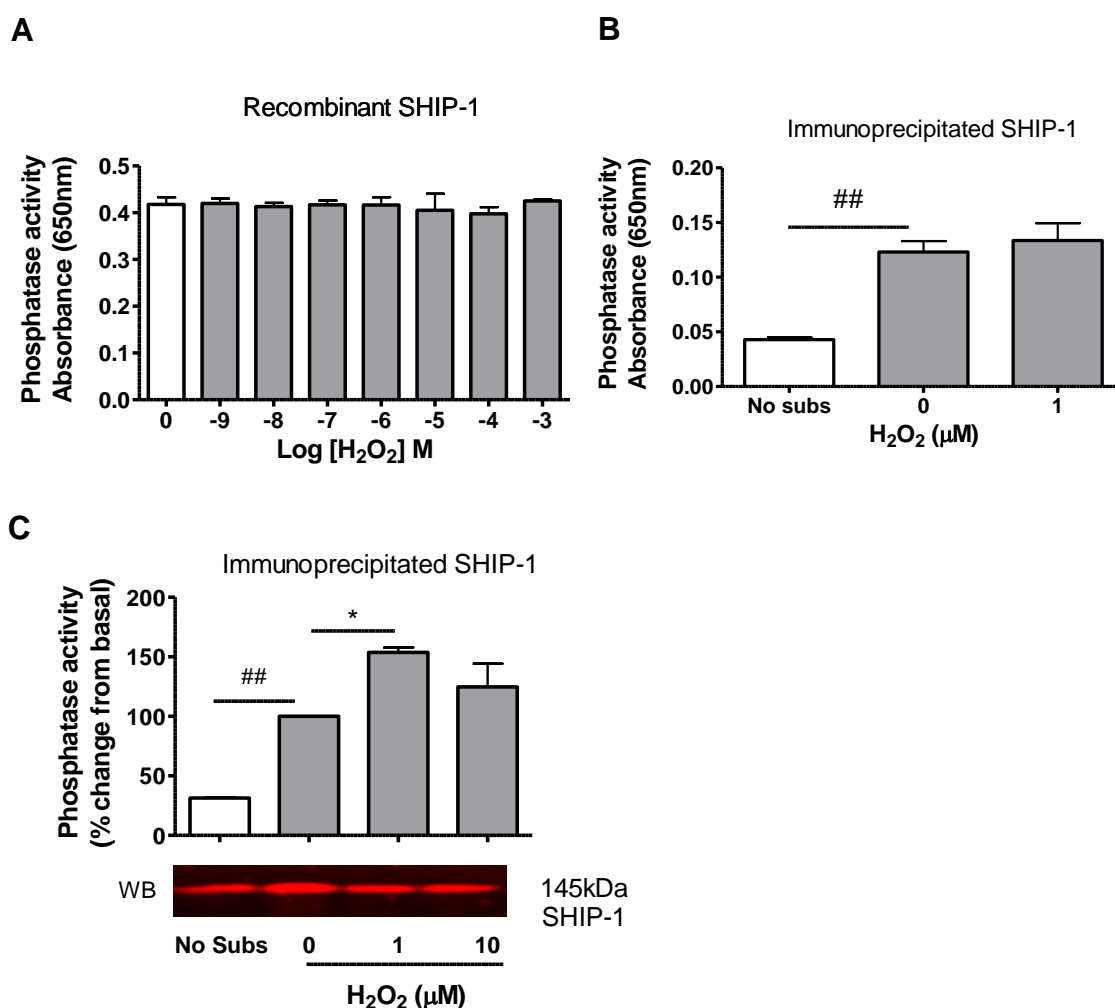


Figure 5.11 Cellular treatment with H₂O₂ enhanced the catalytic ability of SHIP-1, but had no effect upon recombinant or immunoprecipitated SHIP-1. (A) 1μM full length recombinant SHIP-1 or (B) the absorbance of immunoprecipitated SHIP-1 alone (no subs) or treated with 1μM of H₂O₂ for 30 minutes at 37°C. (C) SEB-activated T lymphocytes were treated with 1 or 10μM H₂O₂ for 30 minute and then SHIP-1 was immunoprecipitated. 100μM inositol(1,3,4,5)tetrakisphosphate (IP₄) was added to the SHIP-1 for 30 minutes at 37°C and then 100μl malachite green solution was added. The level of free phosphate was quantified by reading the absorbance at 650nm. Level of SHIP-1 protein was quantified using Western Blotting, using fluorescently tagged secondary antibody and fluorescence detected using LiCor Image analyser. Statistical significance was determined by one way-ANOVA with Dunnett's post-test where *p<0.05, **p<0.01 as compared to control or ##p<0.01 as compared to no substrate control

5.5 ROS manipulation on the surface expression of CXCR3

ROS manipulation was previously shown to alter T lymphocyte migration and actin polarisation to CXCL11 (Chapter 4), processes which require signalling through the chemokine receptor CXCR3. Therefore, the effect of ROS manipulation upon the surface expression of CXCR3 was investigated. A 30 minute treatment of ROS manipulators was used in an attempt to limit any transcriptional changes which would be unlikely to occur within this short timeframe, and instead focused upon determining the effect of ROS upon receptor trafficking.

5.5.1 Exogenous application of hydrogen peroxide (H₂O₂) causes down-regulation of cell surface CXCR3 expression but MnTBAP chloride had no effect

Firstly, SEB-activated T lymphocytes were treated with increasing concentrations of H₂O₂ for 30 minutes and CXCR3 surface expression was determined by flow cytometry. H₂O₂ treatment caused a concentration dependent reduction in CXCR3 expression (Figure 5.12 A) with an IC₅₀ 2µM. Thus, H₂O₂ significantly decreases the expression of CXCR3 at concentrations which affect migration but are not cytotoxic.

The effect of superoxide dismutase mimetic, MnTBAP chloride was also examined upon CXCR3 expression in order to determine whether increasing intracellular H₂O₂ would have the same effect as exogenous application of H₂O₂. However, inconsistently with its ability to decrease migration (Figure 4.12), MnTBAP chloride had no effect upon SEB-activated T lymphocyte CXCR3 expression (Figure 5.12 B).

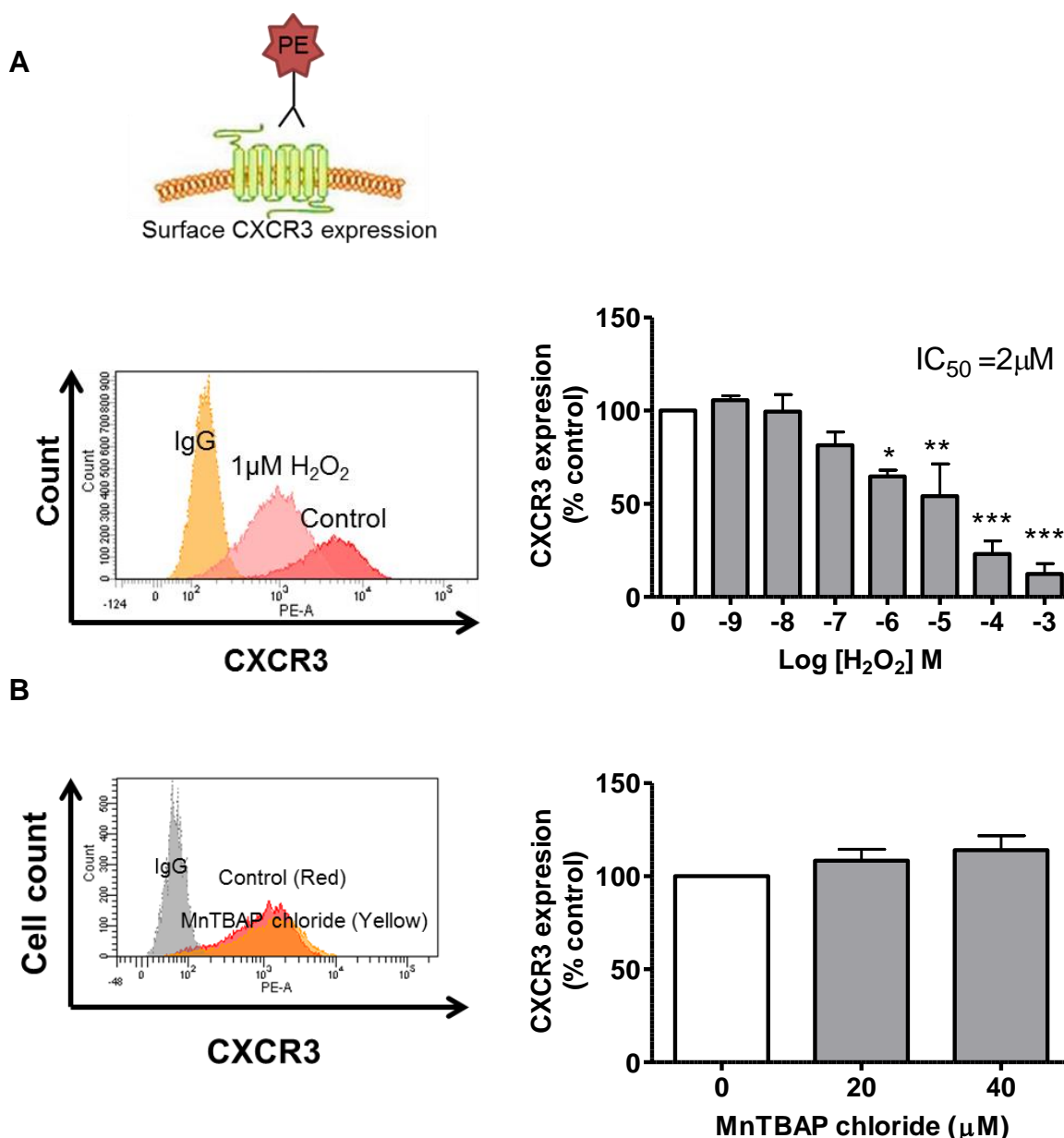


Figure 5.12 H_2O_2 reduced the surface receptor expression of CXCR3 in SEB-activated T lymphocytes, but MnTBAP chloride had no effect. SEB-activated T lymphocytes were washed three times in serum free media and treated with increasing concentrations of (A) H_2O_2 or (B) MnTBAP chloride for 30 minutes. Cells were washed in ice cold PBS, then blocked in 1% BSA for 30 minutes. Cells were then incubated with Alexa-PE-conjugated CXCR3 antibody on ice for 30 minutes. Mean fluorescence intensity per 10,000 cell was measured using flow cytometry. The left panel is a representative FACS plot from a single donor and the right panel is the mean \pm SEM of three independent donors, normalised to the un-treated control. Statistical significance was determined by one way-ANOVA with Dunnett's post-test where * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$ as compared to control.

5.5.2 Effect of dampening the redox environment upon the surface expression of CXCR3

As application of exogenous H₂O₂ down-regulated the surface expression of CXCR3, the effect of dampening ROS upon CXCR3 expression was examined. Firstly, catalase pre-treatment of SEB-activated T lymphocytes caused a small but significant increase in CXCR3 expression (Figure 5.13 A). Catalase evoking a marginal increase in CXCR3 expression is consistent with it increasing migration of CXCL11-induced migration (Figure 4.13). To control for possible impurities in the catalase, the solution was boiled for 10 minutes to deactivate the catalase. Deactivated catalase had no effect upon surface CXCR3 expression (Figure 5.13 B).

Secondly, the redox environment was dampened using diphenyleneiodonium (DPI), a potent NOX inhibitor. Consistently with its inability to alter cell migration (Figure 14.13), DPI had no significant effect upon surface expression of CXCR3 (Figure 5.9 C). Finally, dithiothreitol (DTT) is a reducing agent which has been shown to restore protein activity lost by oxidation (Cleland, 1964). DTT significantly enhanced the surface expression of CXCR3 (Figure 5.13 D), suggesting that oxidation is involved in the internalisation of CXCR3.

5.5.3 Hypoxia significantly enhanced surface receptor expression of CXCR3

Exposure to low oxygen (hypoxia) elevates mitochondrial ROS generated within the cells and appeared to impair SEB-activated T lymphocyte directional migration (Figure 4.14 A). Therefore the effect of a 3 hour exposure to hypoxia was examined to determine whether it had any affect upon the surface expression of CXCR3. Hypoxia un-expectantly enhanced the level of CXCR3 surface expression (Figure 5.14). Although, it was unlikely that 30 minute treatment with H₂O₂ would induce any transcriptional changes in CXCR3 expression, a 3 hour exposure to hypoxia could increase the transcription of CXCR3 resulting in higher surface CXCR3 expression.

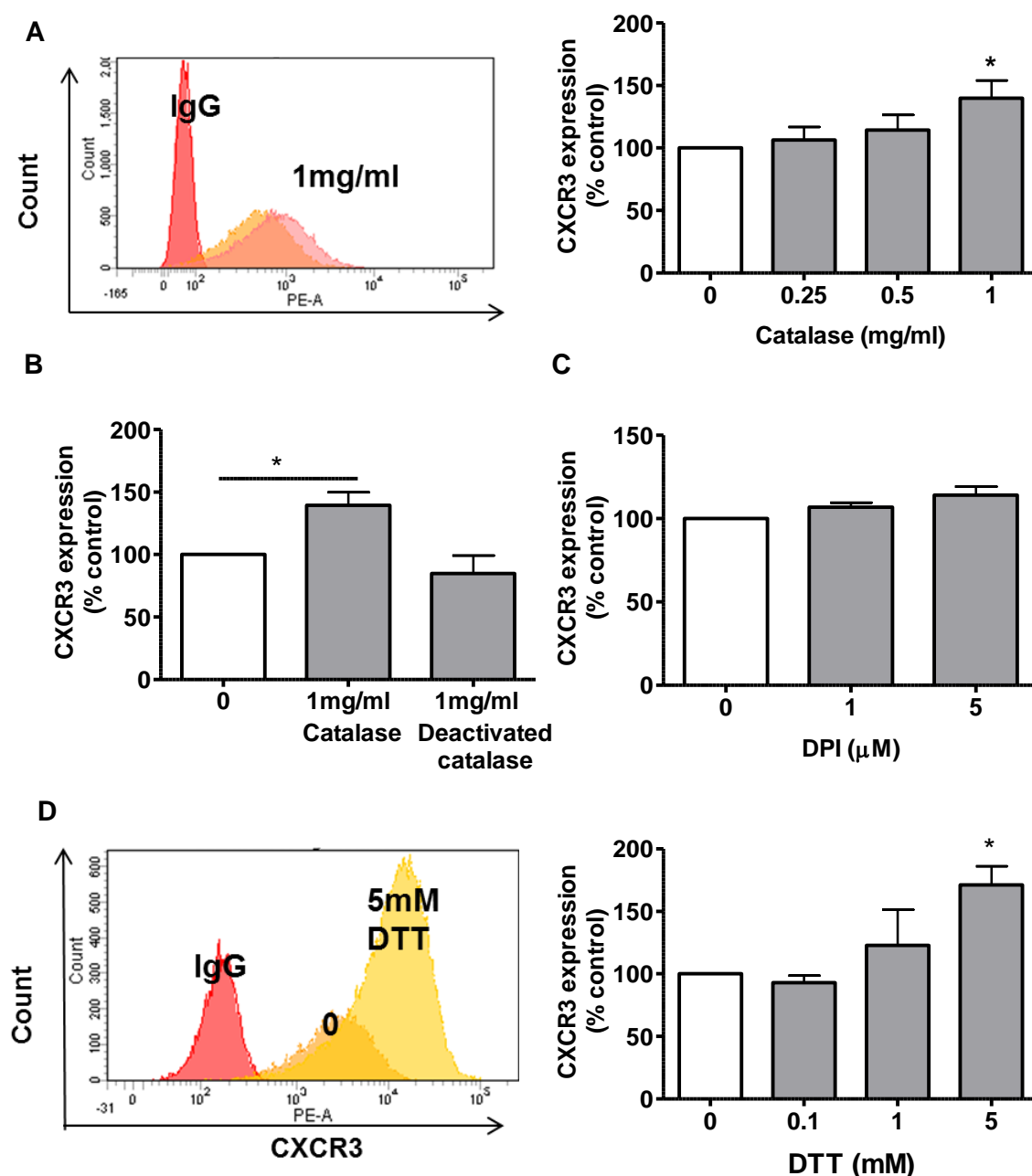


Figure 5.13 Effect of catalase and DPI upon surface receptor expression of CXCR3 in SEB-activated T lymphocytes. SEB-activated T lymphocytes were washed three times in serum free media and treated with increasing concentrations of (A) catalase, (B) deactivated catalase, (C) DPI or (D) DTT for 30 minutes. Cells were washed in ice cold PBS, then blocked in 1% BSA for 30 minutes. Cells were then incubated with Alexa-PE-conjugated CXCR3 antibody on ice for 30 minutes. Mean fluorescence intensity per 10,000 cells was measured using flow cytometry. The left panel is a representative FAC plot for a single donor and the right panel is the mean \pm SEM of three independent donors, normalised to the un-treated control. Statistical significance was determined by one way-ANOVA with Dunnett's post-test where * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$ as compared to control.

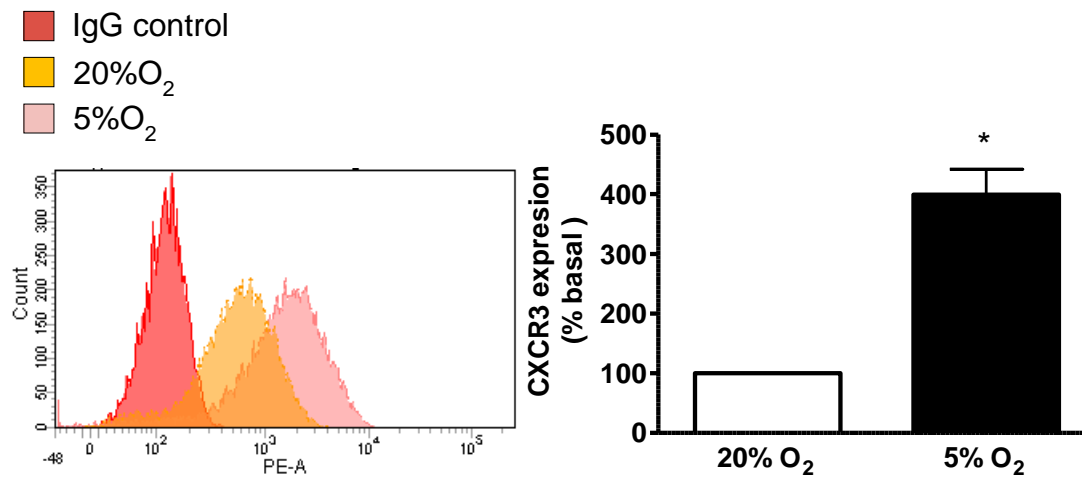


Figure 5.14 Hypoxia significantly enhanced surface receptor expression of CXCR3 in SEB-activated T lymphocytes. SEB-activated T lymphocytes were washed three times in serum free media and placed in either standard cell culture conditions (air: 5% CO₂: 37°C) or a hypoxic chamber (5% O₂: 5% CO₂: 90% N₂ 37°C) for 3 hours. Cells were washed in ice cold PBS, then blocked in 1% BSA for 30 minutes. Cells were then incubated with Alexa-PE-conjugated CXCR3 antibody on ice for 30 minutes. Mean fluorescence intensity per 10,000 cells was measured using flow cytometry. The left panel is a representative FACS plot from a single donor and the right panel is their mean \pm SEM of three independent donors, normalised to the un-treated control. Statistical significance was determined by one way-ANOVA with Dunnett's post-test where * p <0.05, as compared to control.

5.5.4 H₂O₂-induced down-regulation of CXCR3 surface expression requires SFK signalling

As SFK signalling is required for H₂O₂-induced SHIP-1 phosphorylation, the effect of SFK inhibition upon H₂O₂-induced CXCR3 down-regulation was examined. Firstly, SEB-activated T lymphocytes were pre-treated with 1 μ M PP2, a non-selective SFK inhibitor, for 30 minutes. Cells were then exposed to increasing concentrations of H₂O₂ and levels of surface CXCR3 expression observed by flow cytometry. Pre-treatment with PP2 significantly impaired the ability of H₂O₂ to down-regulate CXCR3 expression (Figure 5.15 A), suggesting that CXCR3 down-regulation in activated T lymphocytes requires SFK signalling.

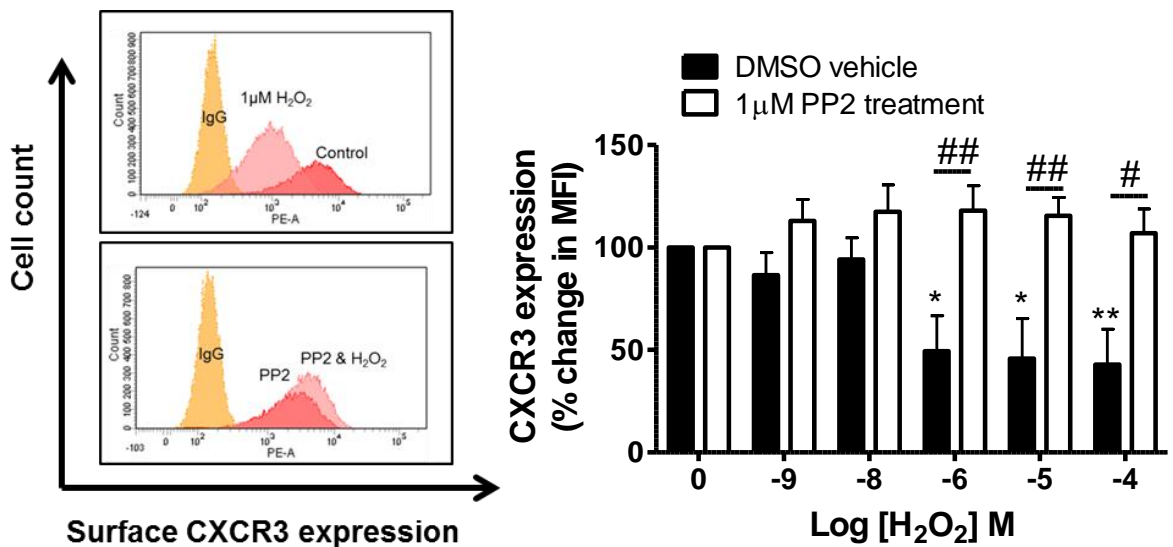


Figure 5.15 SFK inhibitor (PP2) inhibited H₂O₂-induced down-regulation of CXCR3 surface receptor expression in SEB-activated T lymphocytes. SEB-activated T lymphocytes were washed three times in serum free media and treated with (A) PP2 (1 μ M) for 30 minutes then cells were treated with increasing concentration of H₂O₂. Cells were washed in ice cold PBS, then blocked in 1% BSA for 30 minutes. Cells were then incubated with Alexa-PE-conjugated CXCR3 antibody on ice for 30 minutes. Mean fluorescence intensity per 10,000 cells was measured using flow cytometry. The left panel is a representative FACS plot from a single donor and the right panel is the mean \pm SEM of three independent donors, normalised to the untreated control. Statistical significance was determined by two-way ANOVA with Bonferroni's post-test where * p <0.05 or ** p <0.01 as compared to control and # p <0.05, ## p <0.01 comparison with H₂O₂.

5.5.5 H₂O₂-induced down-regulation of CXCR3 surface expression does not require PI3K signalling

H₂O₂ has been shown to activate both PI3K and SHIP-1 signalling. Thus, PI3K signalling could also play a part in H₂O₂ decreasing CXCR3 expression. To examine whether the PI3K signalling was required for H₂O₂-induced decrease in CXCR3 surface expression, SEB-activated T lymphocytes were pre-treated with 1µM ZSTK474 a pan PI3K kinase inhibitor for 30 minutes before exposure to increasing concentrations of H₂O₂. Pre-treatment with PI3K inhibitor had no effect upon H₂O₂ to down-regulate CXCR3 expression (Figure 5.16 A). This implies CXCR3 down-regulation does not require PI3K signalling, and is consistent with the observation that SHIP-1 activation and inhibition did not affect CXCR3 expression (Figure 3.13).

5.5.6 CXCL11-induced down-regulation of CXCR3 expression does not require SFK

Ligand-induced desensitization and internalization of chemokine receptors is a critical mechanism by which GPCR signalling is regulated. CXCL11 is known to down-regulate the surface expression of CXCR3 at the surface membrane by enhancing receptor recycling and degradation (Meiser *et al.*, 2008). Interestingly H₂O₂-induced down-regulation of CXCR3 surface expression required SFK signalling; therefore the next step was to establish whether CXCL11 also required SFK signalling to reduce expression of CXCR3.

SEB-activated T lymphocytes were pre-treated with 1µM PP2, a non-selective SFK inhibitor, or 1µM Lck inhibitor 1188890-30-3 for 30 minutes. Cells were then exposed to increasing concentrations of CXCL11 and levels of surface CXCR3 expression observed by flow cytometry. CXCL11 evoked a concentration dependent reduction in the expression of CXCR3, however neither SFK nor Lck inhibition had any effect upon CXCL11-induced down-regulation of CXCR3 (Figure 5.16 B). This suggests that H₂O₂ and CXCL11 induce distinct intracellular signalling to internalise the CXCR3 receptor.

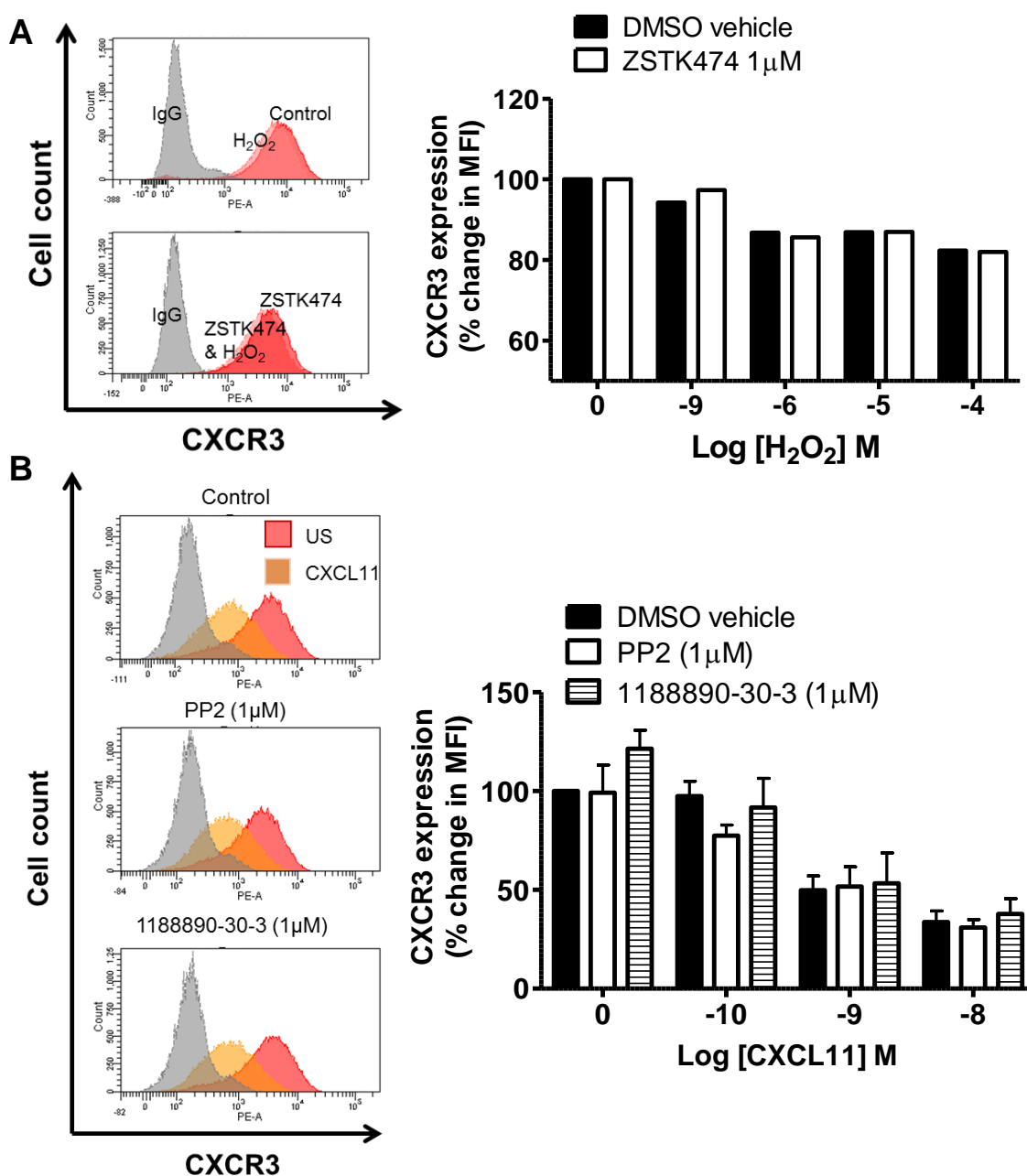


Figure 5.16 PI3K inhibition does not affect H_2O_2 -induced down-regulation of CXCR3 and CXCL11-induce downregulation of CXCR3 does not require SFK (A) SEB-activated T lymphocytes were washed three times in serum free media and treated with ZSTK474 (1 μM) for 30 minutes, followed by treatment with increasing concentration of H_2O_2 . Cells were washed in ice cold PBS, then blocked in 1% BSA for 30 minutes. Data are (left panel) a representative FAC plot and (right panel) the mean \pm SEM normalised to untreated control from two independent donors. SEB-activated T lymphocytes were washed three times in serum free media and treated with (A) PP2 (1 μM) or (B) 1188890-30-3 (1 μM) for 30 minutes then cells were treated with increasing concentration of CXCL11. Cells were then incubated in either IgG control antibody or Alexa-PE-conjugated CXCR3 antibody on ice for 30 minutes and washed twice in ice cold PBS. Mean fluorescence intensity per 10,000 cells was measured using flow cytometry. Data are (left panel) a representative FACS plot and (right panel) the mean \pm SEM minus IgG control, normalised to untreated control from three independent donors.

5.6 H₂O₂ induces Ca²⁺ elevation in SEB-activated T lymphocytes

An important cellular signal involved in T cell activation and migration is elevation of intracellular Ca²⁺ (Negulescu *et al.*, 1994). There have been many reports across mammalian cells that H₂O₂ can cause mobilisation of intracellular Ca²⁺ (Korzets *et al.*, 1999). To determine whether H₂O₂ induces intracellular Ca²⁺ release in human T lymphocytes, cells were loaded with a Ca²⁺ sensitive reporter Fluo-4, and fluorescence was measured over time using a plate reader to measure intracellular Ca²⁺.

5.6.1 H₂O₂-induced Ca²⁺ elevation is unaffected by removal of extracellular Ca²⁺

SEB-activated T lymphocytes were treated with increasing concentration of H₂O₂ and level of Ca²⁺ measured over time. 1mM and 10mM H₂O₂ evoked a significant increase in intracellular Ca²⁺ with an initial steep rise in Ca²⁺ until a stable plateau was reached (Figure 5.17 A). High concentrations of H₂O₂ immediately induce Ca²⁺ mobilisation within T lymphocytes.

To verify that the H₂O₂-induced Ca²⁺ response is due to oxidation and to test whether it is a reversible response, the ability of dithiothreitol (DTT) to reverse the Ca²⁺ rise was examined. DTT is a reducing agent which has been shown to restore protein activity lost by oxidation (Cleland, 1964). Cells were treated with 10mM H₂O₂ until a stable plateau was established, then DTT was added and the reversal measured. 1mM DTT significantly reduced that Ca²⁺ elevation and 10mM DTT completely attenuated the response to resting levels (Figure 5.17 B). This indicates that the H₂O₂ response is reversible and occurs due to oxidation.

Next, the ability of H₂O₂ to induce Ca²⁺ elevation in low Ca²⁺ media was investigated. By using low Ca²⁺ media, extracellular Ca²⁺ was reduced which allowed the proportion of Ca²⁺ from intracellular stores to be established. The removal of extracellular Ca²⁺ did cause a marginal reduction of H₂O₂-induced Ca²⁺ elevation, although not significant (Figure 5.17 C). This suggests that the major Ca²⁺ source is intracellular stores.

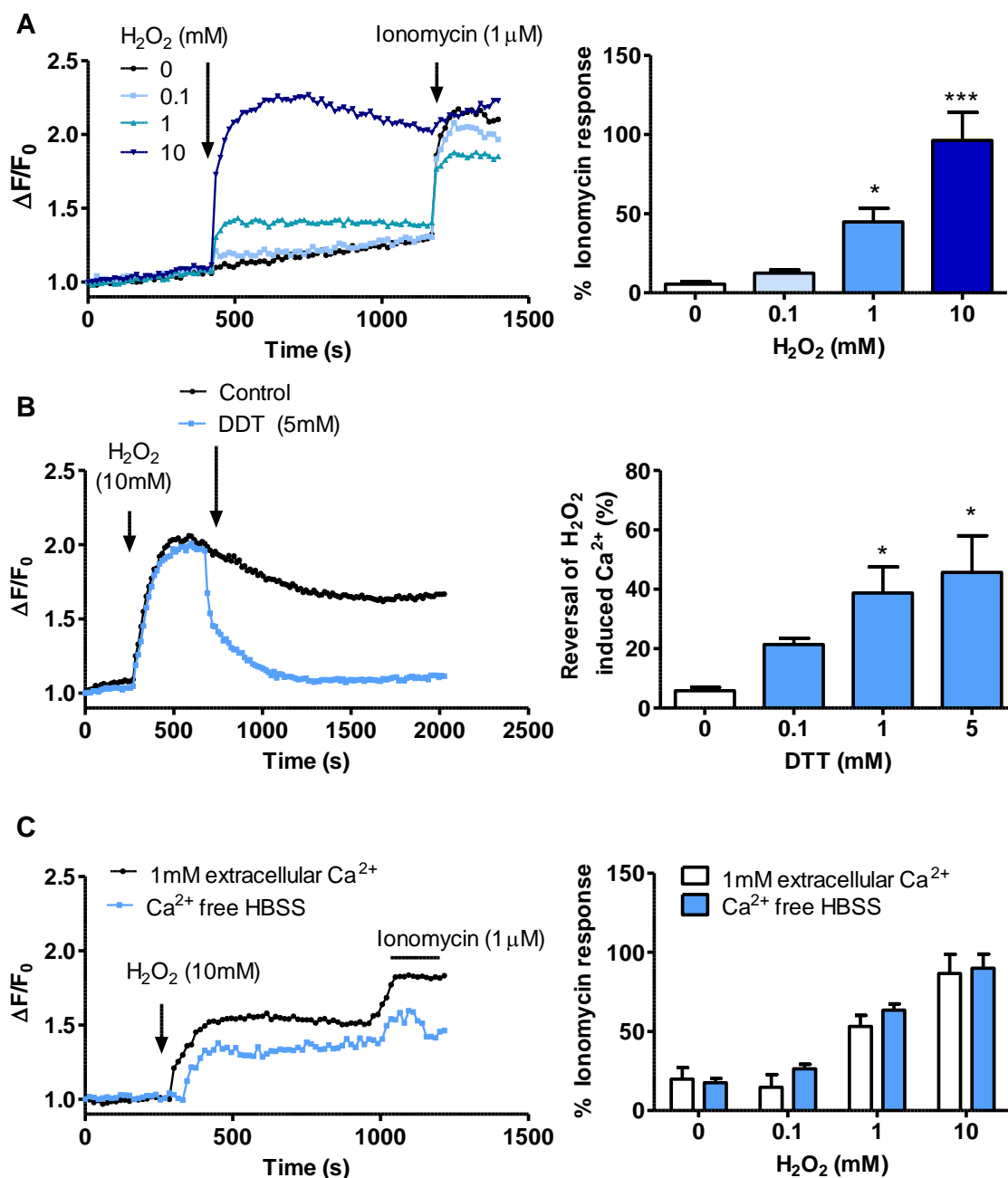


Figure 5.17 H₂O₂-induced intracellular calcium mobilisation is dependent upon oxidation and Ca²⁺ release from intracellular stores in SEB-activated T lymphocytes. SEB-activated T lymphocytes were washed into PBS and loaded for 45 minutes with 1 μ M Fluo-4. Cells were washed 3 times and re-suspended at 1 million cells per ml in Ca²⁺ free-HBSS and (A & B) extracellular Ca²⁺ adjusted to 1mM. Cells were plated in a black 96 well plate and either allowed to rest or treated with (A & C) increasing concentrations of H₂O₂ for 30 minutes or (B) 10mM H₂O₂ (until a stable plateau in Ca²⁺ response was reached) followed by increasing concentrations of DTT. 1 μ M ionomycin was used as a loading control. the left panel is a kinetic trace indicating change in fluorescence from basal ($\Delta F/F_0$) from one representative donor. The right panel is the (A & C) peak change in fluorescence generated by H₂O₂ as a percentage of the peak change generated by ionomycin and (B) % reversal of H₂O₂ response. Data are the mean \pm SEM from averaged duplicates of three independent donors. Statistical significance was tested using a one-way ANOVA with Dunnett's post-test relative to control where * $p < 0.05$ or *** $p < 0.001$.

5.6.2 H₂O₂-induced Ca²⁺ elevation requires PLC but not PI3K, SFK, SYK, MAPK or PKC signalling

H₂O₂-induced Ca²⁺ elevation has been shown to be sensitive to the tyrosine kinase blocker genistein in PBMCs (Korzets *et al.*, 1999). Therefore the effect of inhibiting several cellular kinases was established to determine whether H₂O₂ requires them to initiate Ca²⁺ signalling.

SEB-activated T lymphocytes were treated with the stated concentration each inhibitor for 30 minutes followed by increasing concentrations of H₂O₂ and the level of intracellular Ca²⁺ measured using fluo-4 fluorescence. As shown above, H₂O₂ induces Ca²⁺ elevation at concentrations which were shown to activate Akt, ERK and Src kinase phosphorylation (Figure 5.6). In addition, as SFKs are required for H₂O₂-induced SHIP-1 phosphorylation and CXCR3 down-regulation, the effects of PI3K, SFK and SYK inhibition were investigated. 1µM PI3K (LY294002 and ZSTK474), SFK (PP2) and SYK (picetannol) inhibition had no effect upon H₂O₂-induced Ca²⁺ mobilization (Figure 5.18) and therefore are not required for H₂O₂-induced Ca²⁺ release.

IKK inactivates NFκB and participates in several protein cascades including MAPK. Alongside IKK (TPCA-1α), mitogen-activated protein kinases (p38; SCIO-469α) and mitogen-activated protein kinase kinase (MEK; PD0325901α) inhibition was investigated to determine whether Ca²⁺ release was dependent upon MAPK signalling. IKK, p38 and MEK inhibition had no effect upon H₂O₂-induced Ca²⁺ mobilization (Figure 5.19). This implies MAPK signalling is not involved in H₂O₂-induced Ca²⁺ elevation.

The most common pathway involved in Ca²⁺ mobilisation is phospholipase C (PLC), which hydrolyses the membrane lipid PI(4,5)P₂ into IP₃ and diacylglycerol (DAG). IP₃ binds to the IP₃ receptor, a Ca²⁺ channel which releases Ca²⁺ from the endoplasmic reticulum. PLC inhibitor U73122 significantly reduced the effect of 10mM H₂O₂ to evoke Ca²⁺ mobilization (Figure 5.20 B). Importantly, U73343, an inactive chemical analog of U73122, was used as a negative control and had no effect upon H₂O₂-induced Ca²⁺ mobilization (Figure 5.20 A), hence H₂O₂ requires PLC signalling to maximally elevate intracellular Ca²⁺. DAG activates protein kinase C (PKC) and therefore the effect of PKC inhibition was also determined on H₂O₂-induced Ca²⁺. The PKC inhibitor RO-32-0432 had no effect upon H₂O₂-induced Ca²⁺ mobilization (Figure 5.20 C).

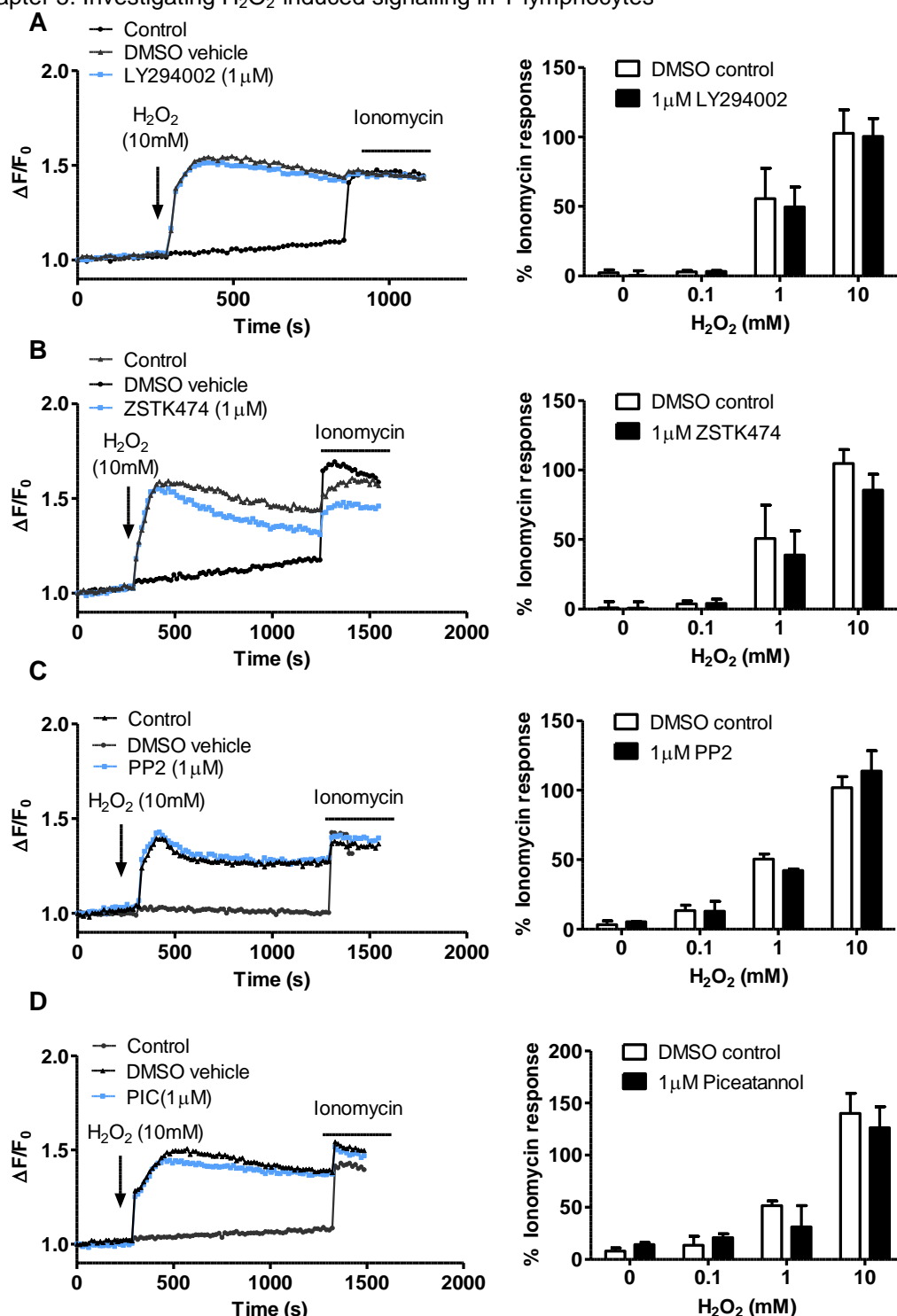


Figure 5.18 PI3K, SFK and SYK inhibition had no effect upon H₂O₂-induced intracellular calcium mobilisation in SEB-activated T lymphocytes. SEB-activated T lymphocytes were washed into PBS and loaded for 45 minutes with 1 μ M Fluo-4. Cells were washed 3 times and re-suspended at 1 million cells per ml in HBSS and extracellular Ca²⁺ adjusted to 1mM. Cells were plated in a black 96 well plate and treated with 1 μ M (A) LY294002, (B) ZSTK474, (C) PP2 or (D) piceatannol, and then treated with increasing concentrations of H₂O₂ for 30 minutes. 1 μ M ionomycin was used as a loading control. Left panel is a kinetic trace indicating change in fluorescence from basal ($\Delta F/F_0$) from one representative donor. The right panel is the percentage of the ionomycin response and is the mean \pm SEM from averaged duplicates of three independent donors. Statistical significance was determined using a two-way ANOVA with Bonferroni post-test relative to control.

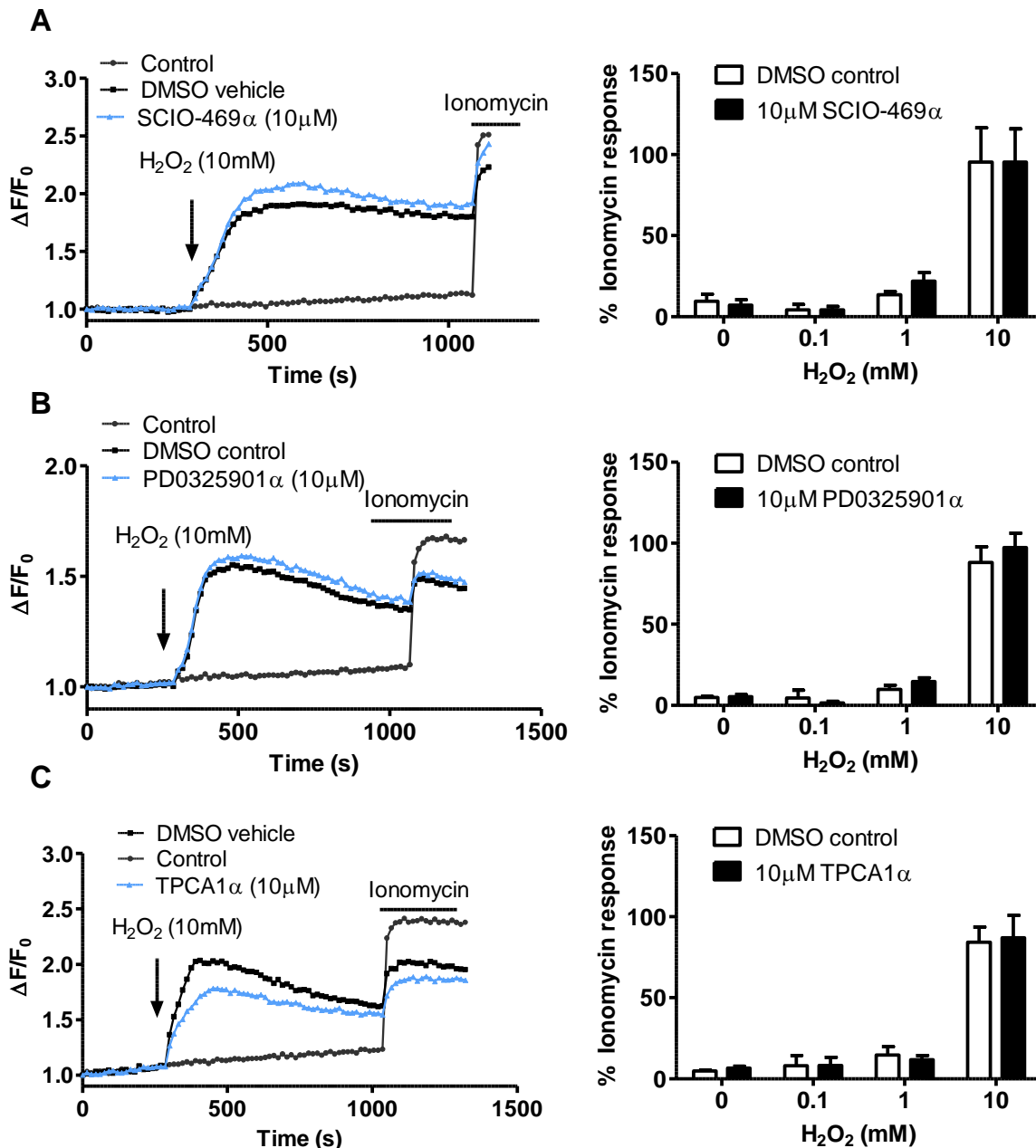


Figure 5.19 p38, MEK and IKK inhibition had no effect upon H₂O₂-induced intracellular calcium mobilisation in SEB-activated T lymphocytes. SEB-activated T lymphocytes were washed into PBS and loaded for 45 minutes with 1μM Fluo-4. Cells were washed 3 times and re-suspended at 1 million cells per ml in Ca²⁺ free-HBSS and extracellular Ca²⁺ adjusted to 1mM. Cells were plated in a black 96 well plate and treated with 10μM (A) SCIO-469α (B) PD0325901α and (C) TPCA1α and then treated with increasing concentrations of H₂O₂ for 30 minutes. 1μM ionomycin was used as a loading control. Left panel is a kinetic trace indicating change in fluorescence from basal ($\Delta F/F_0$) from one representative donor. The right panel is the percentage of the ionomycin response and is the mean \pm SEM from averaged duplicates of three independent donors. Statistical significance was determined using a two-way ANOVA.

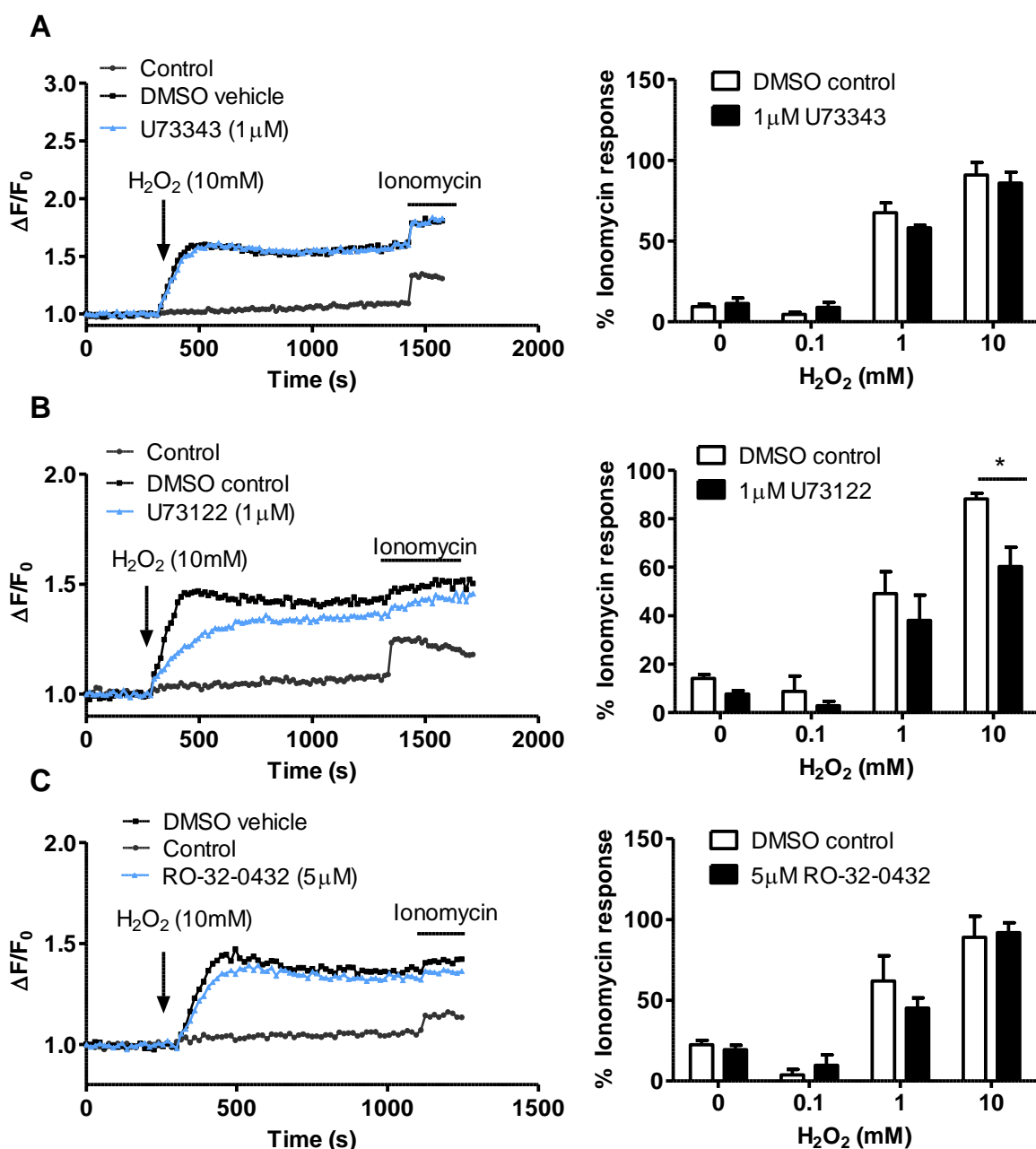


Figure 5.20 PLC but not PKC inhibition significantly inhibited H₂O₂-induced intracellular calcium mobilisation in SEB-activated T lymphocytes. SEB-activated T lymphocytes were washed into PBS and loaded for 45 minutes with 1 μ M Fluo-4. Cells were washed 3 times and re-suspended at 1 million cells per ml in Ca²⁺ free-HBSS and extracellular Ca²⁺ adjusted to 1mM. Cells were plated in a black 96 well plate and treated with 1 μ M (A) U73343 (B) U73122 and 5 μ M (C) RO-32-0432 and then treated with increasing concentrations of H₂O₂ for 30 minutes. 1 μ M ionomycin was used as a loading control. Left panel is a kinetic trace indicating change in fluorescence from basal ($\Delta F/F_0$) from one representative donor. The right panel is the percentage of the ionomycin response and is the mean \pm SEM from averaged duplicates of three independent donors. Statistical significance was tested using a two-way ANOVA with Bonferroni post-test where * p <0.05 relative to untreated control.

5.7 Chapter 5 Results Summary

(a) Small molecule inhibitors upon SEB-activated T lymphocyte migration

- Basal migration requires PI3K, PLC, PKC and SFK signalling
- CXCL11-induced migration requires PI3K, PLC, SFK and SYK signalling
- Inhibition of SFK, SYK or PI3K cannot rescue the migratory defect observed with H₂O₂

(b) Hypoxia

- Exposure to hypoxia significantly inhibits SEB-activated migration to CXCL11
- Exposure to hypoxia for 3 hours significantly enhanced the surface expression of CXCR3

(c) ROS signalling

- High concentrations of H₂O₂ require PLC signalling to induce Ca²⁺ mobilisation
- Oxidative stress induces phosphorylation of Src kinase, Akt and ERK
- Catalase treatment can suppress H₂O₂-induced Akt phosphorylation
- Low concentrations of H₂O₂ require SFK signalling to enhance the phosphorylation and catalytic activity of SHIP-1
- Low concentrations of H₂O₂ down regulated the surface expression of CXCR3, however MnTBAP chloride had no effect
- Catalase marginally increased surface expression of CXCR3, whereas DPI had no effect
- SFK signalling was required to down-regulate the surface expression of CXCR3
- PI3K signalling was not required to down-regulate the surface expression of CXCR3
- CXCL11 does not require SFK signalling to down-regulate the surface expression of CXCR3

5.8 Chapter 5 Discussion

Importance of SFK and SYK in CXCL11-induced migration

SFK members, Lck and Fyn, and SYK, ZAP-70, are known to have a critical role in T lymphocyte development and activation and are recruited and phosphorylate immunoreceptor tyrosine based activation motifs (ITAMs) within CD3 and the zeta chain of the TCR receptor (Salmond *et al.*, 2009). Phosphorylated ITAMs serve as docking sites for SYK to become activated and initiate signalling. However, the signalling roles of SFKs and SYK in T lymphocyte migration are less well understood. The G protein, G α i, has been shown to directly stimulate Src kinase activity (Ma *et al.*, 2000) and Lyn kinase is activated downstream of both CXCR4 in B cells (Ptasznik *et al.*, 2002) and CCR5 in macrophages (Tomkowicz *et al.*, 2006).

Here, the specific contribution of SFK and SYK signalling in SEB-activated T lymphocyte migration towards CXCL11 was examined. It has previously been observed that either genetic silencing of the Lck (Zaman *et al.*, 2008) or ZAP-70 (Ottoson *et al.*, 2001) in Jurkat cells inhibits their migration towards CXCL12. CXCR3 stimulation with CXCL10 has been shown to require both SFK and SYK signalling to induce adhesion to VCAM-1 in Jurkat cells stably expressing CXCR3 (Sun *et al.*, 2014). Interestingly, both SFK and SYK signalling is required for CXCL11-induced directional migration of primary human SEB-activated T lymphocytes (Table 5.1), indicating that CXCL11-induced ligation of the CXCR3 receptor initiates SFK and SYK signalling.

PI3K signalling is required for activated T lymphocyte migration

Activation of PI3K is a robust signalling event, which occurs upon ligation of chemokine receptors and has been reported to be required for directional migration across several cell types (Welf *et al.*, 2012; Gambardella *et al.*, 2013; Wu *et al.*, 2015). Specifically in T lymphocytes, PI3K signalling has been shown to regulate the chemotactic migration of freshly isolated CD4/CD8 T lymphocytes and leukemic T cell lines to CCL19, CCL21 and CXCL12 (Sasaki *et al.*, 2000; Reif *et al.*, 2004). However, it has previously been observed that chemokine-induced migration of activated T lymphocytes is resistant to the pan PI3K inhibitors LY294002 and wortmannin (Smit *et al.*, 2003; Cronshaw *et al.*, 2006). This led to the hypothesis that ex-vivo maintenance and activation/differentiation of the T lymphocytes leads to the acquiring a resistance to PI3K inhibition and undertaking migration using PI3K independent signalling.

Surprisingly, the pan PI3K inhibitor ZSTK474 evoked a concentration dependent reduction in CXCL11-driven migration of activated T lymphocytes which was significant at 10µM. Consistently with the previous studies, the directional migration of activated T lymphocytes was resistant to pan PI3K inhibitor LY294002. In an attempt to understand the distinct outcomes of ZSTK474 and LY294002 upon SEB-activated migration, their molecular pharmacological profile was researched in the literature. LY294002 is a member of the first generation of PI3K inhibitors and is 30 fold less potent than ZSTK474 at inhibiting PI3K isoforms. ZSTK474 is most effective at inhibiting PI3Kδ, whereas LY294002 is most effective at inhibiting PI3Kα (Kong *et al.*, 2007). The IC₅₀ of each drug at inhibiting PI3K isoforms are summarised in Table 1.3. In addition to LY29004 being a less potent inhibitor of PI3K, it has also been shown to have more non-selective effects, with concentrations above 10µM LY29004 inhibiting other signalling enzymes including class II PI3K and PtdIns 4-kinases (Fruman *et al.*, 1998). LY29004 also has activity against mTOR signalling above 5µM (Brunn *et al.*, 1996). Thus, ZSTK474 is more selective and potent than LY294002. Even at 100µM, ZSTK474 only weakly inhibits mTOR (Kong *et al.*, 2007).

As the pan PI3K inhibitor ZSTK474 inhibited migration, the effect of isoform specific inhibitors of PI3K were investigated upon the migration of activated T lymphocytes. Neither the PI3Kγ inhibitor AS-252424 nor PI3Kδ inhibitor IC871114 had any effect upon basal or CXCL11 migration. However, as redundancy often exists between the different isoforms of PI3K (Chaussade *et al.*, 2007; Foukas *et al.*, 2010; Juss *et al.*, 2012), the effect combining AS-252424 and IC871114 was examined. The collective inhibition of both isoforms significantly impaired both basal and CXCL11-induced migration in a similar fashion to ZSTK474, confirming that PI3K signalling is required for directional migration towards CXCL11 and implying that PI3Kγ and PI3Kδ provide compensatory roles. In addition, ZSTK474 was observed to completely attenuate CXCL11-induced Akt phosphorylation, indicating the importance of PI3K in initiating signalling in SEB-activated T lymphocytes.

It is important to note that migration was not completely abrogated with PI3K inhibition, suggesting that although PI3K signalling is not dispensable, activated T lymphocytes may also require PI3K independent signalling pathways to undertake migration. Indeed, it was also shown that CXCL11-induced migration required signalling through phospholipase C (PLC), a PI3K independent pathway involved in T lymphocyte migration (Table 5.1). Phospholipase C (PLC) hydrolyses the membrane lipid PI(4,5)P₂ into IP₃ and diacylglycerol (DAG). IP₃ binds to the IP₃ receptor, a Ca²⁺ channel which releases Ca²⁺ from the endoplasmic reticulum. The PLC inhibitor U73122 significantly

reduced basal and CXCL11-induced migration. Importantly, the negative control U73343, an inactive chemical analog of U73122, had no effect upon CXCL11-induced migration, but surprisingly appeared to have a marginal effect upon basal migration. This suggests that the inhibition of basal migration by U73122 may at least partially occur through a non-selective effect. Interestingly, inhibition of PKC had no effect upon CXCL11 migration, which implies DAG induced signalling is not required for CXCL11-induced migration of activated T lymphocytes.

Compound	Inhibits	Basal migration	CXCL11-induced migration
LY29004	Pan PI3K	↓	-
ZSTK474	Pan PI3K	↓	↓
AS-252424	PI3K γ	-	-
IC871114	PI3K δ	-	-
AS-252424 & IC871114	PI3K γ & PI3K δ	↓	↓
PP2	SFK	↓	↓
Piceatanol	SYK	-	↓
U73343	Inactive PLC	↓	-
U73122	Active PLC	↓	↓
RO-32-0432	PKC	↓	↓

Table 5.1 Signalling involved in basal and CXCL11-stimulated migration of activated T lymphocytes ↓ migration decreased, - migration un-affected

Effect of hypoxia upon T lymphocyte migration and CXCR3 expression

In contrast to standard cell culture conditions which provide 20% oxygen, the human body provides much lower oxygen concentrations, ranging from 16% in the pulmonary alveoli, to 6% in most organs (Semenza, 2001). Oxygen concentrations can be extremely low in pathological sites with low vasculature, such as tumours and sites of infection (Vaupe, 1977; Via *et al.*, 2008). Indeed, dysregulation of O₂ homeostasis is observed in inflammatory and cardiovascular diseases, cancer, cerebrovascular disease, and chronic obstructive pulmonary disease.

Under hypoxic (5% O₂) conditions, mitochondria produce higher levels of ROS from the respiratory complex III (Guzy *et al.*, 2005). This diffuses into the cytosol where it stabilises hypoxia-inducible factor 1, leading to gene transcription and cellular adaption to the hypoxic conditions (Eltzschig *et al.*, 2011). Therefore, in addition to being a relevant environment setting for an activated T lymphocyte, acute exposure to hypoxia presents a method of manipulating the redox environment of the cell.

Emerging evidence suggests that the expression and functions of GPCRs can be controlled by microenvironment triggers. Hypoxia regulates leukocyte migration, suppressing the migration of macrophages and monocytes (Turner *et al.*, 1999; Grimshaw *et al.*, 2001). Indeed, decreased expression of CCR2 has been observed in macrophages associated with the hypoxic environment of human carcinoma (Sica *et al.*, 2000) whilst HIF-1 α expression induced CXCR4 up-regulation in human monocytes, macrophages, endothelial cells and cancer cells (Schioppa *et al.*, 2003). Similarly to macrophages, hypoxia reduced the migration of SEB activated T lymphocytes. Interestingly 3 hours exposure to hypoxia caused a 400% enhancement in CXCR3 expression which could imply that hypoxia is prohibiting the internalisation of the receptor or that HIF-1 α is able to up-regulate total CXCR3 expression in 3 hours. It would be interesting to further this work and investigate the effects of hypoxia on other aspects of human T lymphocytes biology. It is important to note that although 5% O₂ is hypoxic compared to standard cell culture conditions this is likely to be a physiological level or “tissue normoxia” rather than pathological hypoxia. It would be interesting to further reduce the O₂ level to reflect inflammatory hypoxia.

H₂O₂-induced signalling in T lymphocytes

The mechanisms that immune cells use to detect ROS are not well established. It has been proposed the leukocytes could express H₂O₂ sensitive receptors (Niethammer *et al.*, 2009). Alternatively H₂O₂ has been shown to manipulate cell function through entering the cytoplasm and directly modifying intracellular mediators. Unravelling the mechanism through which H₂O₂ signals is vital for understanding its role in T lymphocyte biology. Firstly, high concentrations of H₂O₂ impair T lymphocyte proliferation, viability and actin regulation. This section focuses on discussing the effect of high concentrations of H₂O₂ on cellular signalling.

(a) H₂O₂ induces Thiol modifications to increase cytosolic Ca²⁺

High concentrations of H₂O₂ induced an immediate and sustained mobilisation of Ca²⁺ in SEB-activated T lymphocyte, consistent with reports that oxidative stress causes an increase in intracellular Ca²⁺ mobilisation in various mammalian cells (Krippeit-Drews *et al.*, 1995; Herson *et al.*, 1999; Wang *et al.*, 1999; Colston *et al.*, 2002; Zimmerman *et al.*, 2005; Sato *et al.*, 2009). Several different mechanisms and types of Ca²⁺ signalling have been indicated in these cells. Therefore, it was important to determine the mechanism behind ROS-induced Ca²⁺ elevation in human T lymphocytes. Extremely high concentrations of H₂O₂, which are known to affect T cell viability over a longer period, were assumed to cause toxic, irreversible modification of Ca²⁺ homeostasis. However, basal morphological changes were unremarkable after the T lymphocytes were exposed to high (100µM) of H₂O₂ for 30 minutes (Figure 4.20) and ligand-induced Ca²⁺ elevation was unaffected by 30 minute pre-treatment of 100µM H₂O₂ (Figure 4.5 & 4.18). Moreover, the elevation of cytosolic Ca²⁺ was eliminated by the addition of dithiothreitol (DTT). DTT reduces the disulphide bond within the target protein or between protein cysteine thiol and low-molecular-mass thiols such as glutathione. Therefore, H₂O₂ may exert its action by the formation of disulphide bonds.

Although redox homeostasis is tightly maintained by antioxidants, H₂O₂ is known to oxidise lipids and proteins in response to ligand stimulation. In T lymphocytes, ROS is produced from a variety of stimuli, including mitogens (Williams *et al.*, 1996), viral and bacterial superantigens (Weber *et al.*, 1995; Hildeman *et al.*, 1999), TCR peptide agonists (Devadas *et al.*, 2002; Kwon *et al.*, 2003) and CXCL12 (Hara-Chikuma *et al.*, 2012). I have shown that low concentrations of ROS are required for human T lymphocyte survival and proliferation. Thus, H₂O₂ could cause a reversible modification of proteins to trigger positive Ca²⁺ signalling required for cellular survival.

(b) Origin of Ca²⁺ rise induced by H₂O₂

Previous studies have reported that oxidative stress-induced cytosolic Ca²⁺ elevation is distinct in different cell types, with multiple mechanisms reported including release from the endoplasmic reticulum (ER), mitochondria and/or Ca²⁺ influx (Colston *et al.*, 2002; Zimmerman *et al.*, 2005; Nakamura *et al.*, 2009). Extracellular Ca²⁺ influx is not required in H₂O₂-induced Ca²⁺ elevation in human SEB activated T lymphocytes, since the omission of extracellular Ca²⁺ did not abolish the Ca²⁺ increase. Cytosolic Ca²⁺ elevation in human T lymphocytes occurs from intracellular sources. Possible sources include the ER and mitochondria. Ca²⁺ may also be liberated from non-ER and non-mitochondrial Ca²⁺ pool, such as the endosomes and golgi apparatus (Rizzuto *et al.*, 2006) or if due to necrosis from calcium bound to annexin V (Cuschieri *et al.*, 2005). Further experiments using thapsigargin, a sarco/endoplasmic reticulum Ca²⁺ ATPase to deplete the ER or FCCP, a mitochondria uncoupler could help determine the intracellular location of the elevated Ca²⁺.

It is interesting to note that our results are in disagreement with Bogeski *et al.* 2010. Here, primary T_H cells from human donors respond to acute H₂O₂ by immediate increases in intracellular Ca²⁺, however was caused by the opening non-selective cation channels (such as redox sensitive TRPM). Interestingly, T lymphocyte differentiation shifted the activating EC₅₀ values from 130 to 740 μM, indicating that naïve T lymphocytes appear to be more sensitive to ROS (Bogeski *et al.*, 2010). This disparity could be due to the use of different T lymphocyte sub-types; specifically they used T helper cells, whereas I used a mixed population.

(c) PLC inhibition reduced H₂O₂ induced Ca²⁺ increase

H₂O₂ -induced Ca²⁺ elevation in peripheral blood mononuclear cells (PBMC) is sensitive to the tyrosine kinase inhibitor, genistein (Korzets *et al.*, 1999), indicating that intracellular tyrosine kinase co-ordinates Ca²⁺ elevation in PBMCs. In addition, H₂O₂ has been observed to act directly upon inositol 1,4,5-trisphosphate receptors to activate Ca²⁺ release in endothelial cells (Zheng *et al.*, 2005). Here, inhibition of kinases PI3K, SFK, SYK, PKC and MAPK had no effect upon this H₂O₂ driven response, suggesting their signalling was not required to stimulate cytosolic Ca²⁺ elevation. However, PLC signalling was shown to be required for maximal Ca²⁺ elevation (Figure 5.20). PLC has previously been shown to be redox sensitive in macrophages (Liu *et al.*, 2007).

Perspective – Effect of high concentrations of H₂O₂ on T lymphocytes

ROS species are known to induce bifurcate responses within cells, with high concentrations evoking cell death and low concentration required for cell survival, signalling and proliferation. Here, high concentrations of H₂O₂ activate cellular signalling including PI3K, MAPK, Src (discussed below) and PLC. However, although these are positive signalling pathways that are known to be required for T cell survival and proliferation, it is likely that oxidative stress overwhelms the cellular antioxidants causing oxidative damage (Steinberg, 2013) resulting in the dysregulation of actin, decreased proliferation and survival of T lymphocytes (Figure 5.21).

Consistently with the bifurcate roles, dampening the redox environment also decreases human T lymphocyte survival and proliferation. Interestingly, quantification of thiol oxidation in cells has indicated that oxidation events are not equal across different cellular organelles (Balaban *et al.*, 2005; Go *et al.*, 2008). Instead oxidation events occur in cellular compartments to co-ordinate precise cellular signalling. I hypothesize that under physiological conditions localised ROS in cellular compartments may accumulate to high concentrations to signals through similar positive signalling pathways required for cell proliferation and survival without overwhelming cellular antioxidants and evoking cell death (Figure 5.21).

New methods for studying redox compartmentalization have recently become available, with probes which now permit the observation of rapid redox changes in real time and within single organelles (Belousov *et al.*, 2006; Woolley *et al.*, 2013; Ezerina *et al.*, 2014). Development of these novel tools to measure compartmentalized ROS formation and redox status, will allow for better characterization of ROS signals in compartments and microdomains (Kaludercic *et al.*, 2014).

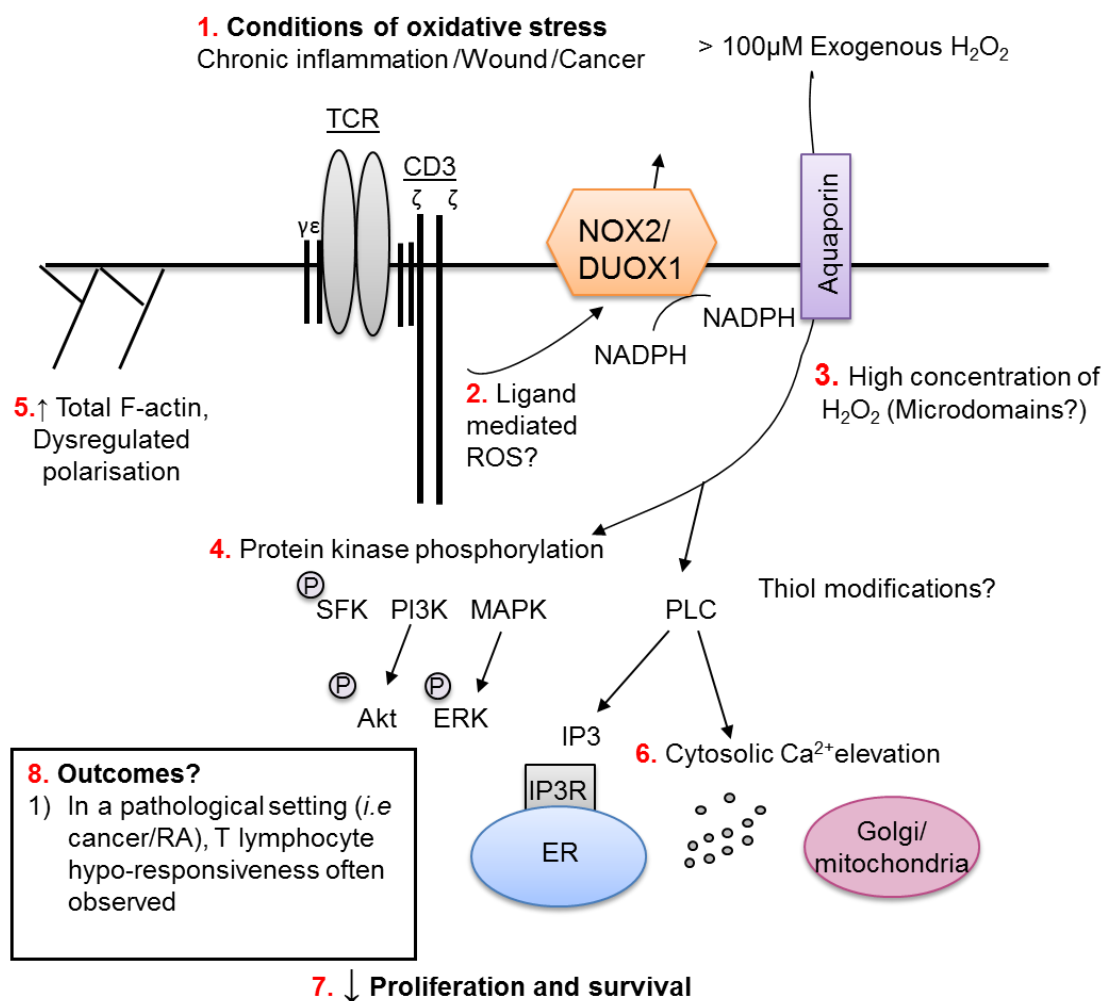


Figure 5.21 Model of oxidative stress-induced signalling in human SEB activated T lymphocytes. This model is based on the results of this chapter and chapter 4 and the findings of (Kwon *et al.*, 2003; Kashiwada *et al.*, 2006) **1)** Several pathologies result in accumulation of high levels of ROS including wounds, chronic infection and cancer exposing T lymphocytes to oxidative stress. **2)** TCR stimulation also produces H₂O₂ through NOX2/DUOX enzymes, which are known to be expressed on the cell membrane and membranes of cellular organelles. Thus, ROS accumulates in microdomains to regulate precise cell signalling. Mitochondria also produce high levels of ROS, especially under hypoxic conditions. **3)** Exposure to >100μM exogenous H₂O₂ places cell under oxidative stress. H₂O₂ enters the cell through aquaporin channels **4)** >100μM H₂O₂-induces the phosphorylation of Src, Akt and ERK, however oxidation might occur at lower concentrations. **5)** >100μM alters actin polarisation and polymerisation, although basal cell morphology is unaltered **6)** >100μM H₂O₂ requires PLC to stimulate intracellular Ca²⁺ from an unknown intracellular source (possibly ER, golgi or mitochondria) **7)** >100μM H₂O₂ evokes oxidative stress which culminates in reduced T lymphocyte proliferation and survival. **8)** Proposed outcomes of exposure to high ROS.

Unravelling the mechanism of H₂O₂-induced migratory defect

Secondly, in order to unravel the mechanisms of H₂O₂-induced migratory defect, the effect of H₂O₂ upon was examined in signalling pathways that are (1) important in cellular migration and (2) have previously been indicated as being redox sensitive, including PI3K, SHIP-1, MAPK and SFK signalling (Truong *et al.*, 2013). In addition, it was thought prudent to investigate the effect of ROS manipulation upon the chemokine receptor CXCR3.

PI3K signalling

Firstly, the asymmetric distribution of PI3Ks product, PI(3,4,5)P₃ at the front of the cell is a hallmark of cell polarisation (Wang *et al.*, 2002a; Wang, 2009). Earlier I described the importance of PI3K in the directional migration of SEB-activated T lymphocytes. Redox inactivation of PTEN, a negative regulator of the PI3K pathway signalling pathway has been previously described and occurs by the formation of a disulphide bond between Cys 124 residue of the active site and Cys71 (Lee *et al.*, 2002; Kwon *et al.*, 2004). High concentrations of H₂O₂ increased Akt phosphorylation in SEB activated T lymphocytes, indicating that H₂O₂ can manipulate PI3K-dependent signalling. However, PI3K inhibition was able to completely attenuate the accumulation of Akt phosphorylation, which implies that H₂O₂ activates PI3K to enhance Akt phosphorylation, rather than inhibiting PTEN (Figure 5.21).

H₂O₂ signalling through PI3K to phosphorylate Akt has been previously described in several other cells types including vascular smooth muscle cells (Ushio-Fukai *et al.*, 1999) and Jurkat cells (Lahair *et al.*, 2006). Interestingly, direct oxidation of Akt has also been described at several specific cysteine residues (Wani *et al.*, 2011), in particular a disulphide bond formed between Cys297 and Cys311, resulting in increased association of Akt with the negative regulatory phosphatase PP2A and dampened Akt signalling (Murata *et al.*, 2003; Durgadoss *et al.*, 2012).

MAPK signalling

Mitogen-activated protein kinases (MAPKs) play crucial roles in cell migration, with ERK important in phosphorylating myosin light chain kinase (MLCK), calpain and FAK (Huang *et al.*, 2004). The MAPK/ERK pathway has also been previously suggested to be redox responsive through inactivation of its regulatory phosphatases (Lee *et al.*, 2003). In addition, there is some evidence of H₂O₂ directly activating MAPKs (Galli *et al.*, 2008), with low levels of H₂O₂ oxidising cysteine residues in ERK2 leading to

conformational changes and increased binding to MEK1/2 whilst oxidative stress inhibits MEKK1 by site specific glutathionylation within the ATP binding domain (Cross *et al.*, 2004). Here, H₂O₂ enhanced the phosphorylation of ERK1/2, although this was only observed at a very high concentration of H₂O₂ (Figure 5.21).

The concentrations of H₂O₂ that evoked phosphorylation of Akt and ERK were likely to place cells under oxidative stress as these concentrations are cytotoxic to SEB-activated T lymphocytes after 24 hour exposure (Figure 4.2). PI3K and MAPK/ERK signalling cascades are also important cellular survival mechanisms. Thus, PI3K and MAPK activation could be a survival signal following oxidative stress. Hypothetically, it is also possible that lower concentrations of H₂O₂ oxidise cysteine residues within signalling proteins which alters their conformation structure and activity without resulting in the phosphorylation of the proteins. Determining whether protein activation through oxidation always results in phosphorylation (a more understood mechanism of protein regulation) would be really interesting to study further. Oxidation has been shown to trigger dimerization or oligomerisation through disulphide bond formation or tag the protein for trafficking or sequestrations, thus indirectly removing it from the vicinity of its target, perhaps exposing it to novel binding partners (Corcoran *et al.*, 2013).

SHIP-1 signalling

H₂O₂ inactivates the phosphatase PTEN (Lee *et al.*, 2002; Kwon *et al.*, 2004) and therefore it has been presumed that it also inactivates SHIP-1, another negative regulator of PI3K signalling. However, the direct effect of H₂O₂ on SHIP-1 catalytic has not been investigated. Therefore, our finding that low concentrations of H₂O₂-induced the phosphorylation and enhanced the catalytic activity SHIP-1 was unexpected. However, SHIP-1 has previously been shown to have a role in protecting cells against H₂O₂ mediated cell death (Gloire *et al.*, 2006), which could possibly be due to it activating signalling down-stream of SHIP-1. SHIP-1 activation could also be a possible mechanism by which H₂O₂ inhibits migration (Figure 5.22), as SHIP-1 activation severely impedes the migration of SEB-activated T lymphocyte migration in Chapter 3.

CXCR3 receptor internalisation

Another mechanism by which H₂O₂ could decrease the migration of T lymphocytes is by regulating the level of chemokine receptor internalisation. Receptor internalisation decreases the amount of available receptor on the cell surface, thus attenuating receptor mediated signalling (Lefkowitz, 1998; Morris *et al.*, 1999) and migration. Chemokines such as CXCL11 cause receptor internalisation to regulate their signalling. Interestingly, H₂O₂ significantly decreases the cell surface expression of CXCR3 in human activated T lymphocytes and dampening the redox environment using catalase resulted in a marginally enhanced surface expression. Thus, H₂O₂ could inhibit T lymphocyte migration by decreasing the surface expression of CXCR3, preventing the cell from detecting and signalling to CXCL11. Both the H₂O₂-induced decrease and catalase-induced increase in CXCR3 surface expression were observed after a 30 minute treatment with H₂O₂, therefore they are unlikely to be changes in the overall expression of the receptor and are likely due to internalisation of the receptor.

Meiser *et al.* observed that even 3 hours after treatment with CXCL11, only 70-80% expression of CXCR3 was re-established. They determined that CXCR3 is degraded following receptor internalisation and has to be replenished on the membrane by *de novo* synthesis (Meiser *et al.*, 2008). This requirement for *de novo* receptor synthesis for expression of CXCR3 may allow for a larger window to observe the effect upon CXCR3 internalisation, however CXCL11 and H₂O₂ might internalise the receptor through completely independent mechanisms.

The regulation of CXCR3 surface expression by H₂O₂ is an important observation as CXCR3 has been indicated in playing a central role in the induction and perpetuation of several human inflammatory disorders including atherosclerosis (Mach *et al.*, 1999), autoimmune diseases (Sorensen *et al.*, 1999), transplant rejection (Hancock *et al.*, 2000) and viral infection (Liu *et al.*, 2000). Thus, the finding that oxidation has a role in regulating surface expression may be an important consideration in the treatment of these diseases.

SFK signalling

To further understand the mechanism by which H₂O₂ is detected by immune cells, I was particularly interested in determining whether SFKs act in the same signalling pathway as H₂O₂ in human SEB activated T lymphocytes. SFK member Lyn has been indicated as a redox sensor involved in early neutrophil recruitment to H₂O₂ at wounds. Mutation of a single conserved cysteine residue to alanine at position Cys466 abolished the ability of Lyn to be oxidised by H₂O₂. This mutation resulted in an inability of the cell to detect and migration towards the H₂O₂ gradient (Yoo *et al.*, 2011).

Cys466 is highly conserved across species (zebrafish Lyn, Drosophila Src-42A and human Lyn) thus oxidation-dependent activation may be a general method of activation for all SFK members, however this has not been fully established. Early evidence suggested that nitric oxide (NO)-releasing agents enhanced the catalytic activity of Src tyrosine kinase through direct oxidation of the kinase (Akhand *et al.*, 1999; Minetti *et al.*, 2002). Subsequently, a number of redox sensitive cysteine residues were identified in Src which are important for the enzymes activity (Senga *et al.*, 2000). Lck and Fyn are expressed in T lymphocytes, were confirmed to contain the critical cysteine 466 residue and Lck has been reported to have redox sensitivity (Nakamura *et al.*, 1993).

The phosphorylation of c-Src at Tyr 416 was assessed as a marker of H₂O₂ ability to activate SFKs in T lymphocytes. The c-Src antibody is likely to cross react with T lymphocyte SFKs, Fyn and Lck. c-Src phosphorylation was not observed until high concentration of H₂O₂. However, as described above it is still possible that oxidation could alter the kinase activity without enhancing phosphorylation. For instance, Giannoni *et al.* observed that ROS exposure caused a disulphide bond formation between Cys245 and Cys487 which enhanced the enzymes activity (Senga *et al.*, 2000; Giannoni *et al.*, 2005).

Excitingly, SFK signalling was required for H₂O₂-induced SHIP-1 phosphorylation in SEB-activated T lymphocytes. This is consistent with how SHIP-1 is phosphorylated down-stream of receptor signalling. SFK kinase Lyn has previously been shown to be required for FcγRIIb-induced phosphorylation of SHIP-1 in B lymphocytes (O'Neill *et al.*, 2011). However, Lyn kinase is not expressed in T lymphocytes (Korade-Mirnic *et al.*, 2000; Scapini *et al.*, 2009), which implies redundancy between different SFK members to phosphorylate SHIP-1. This provides evidence to connect oxidative signalling and SFKs in common pathway in human T lymphocytes and also implies that SFKs are activated by much lower concentration of H₂O₂ than observed to phosphorylate c-Src.

Genestein, a tyrosine kinase inhibitor, has no effect upon CXCL11-induced CXCR3 internalization (Sauty *et al.*, 2001) suggesting that tyrosine kinases are not required in internalisation of CXCR3 upon activation with their ligand. Consistently, SFK inhibition had no effect upon CXCL11-induced internalisation of CXCR3 in SEB activated T lymphocytes. However, SFK inhibition completely attenuated the ability of H₂O₂ to decrease the surface expression of CXCR3. It appears that agonist ligation and oxidative signalling induce distinct pathways to down-regulate CXCR3 surface expression. Consistently to SHIP-1 phosphorylation, H₂O₂ requires SFKs to regulate CXCR3 internalisation.

Finally, inhibition of SFK, SYK and PI3K could not rescue the migratory defect observed with H₂O₂. Instead, all three signalling pathway were required for CXCL11-driven migration. Hypothetically, if H₂O₂ was acting independently of SFK, SYK and PI3K signalling, their inhibition should have produced an additive suppression of migration. SFK signalling appears to play a central role in regulating H₂O₂-induced repression of activated T lymphocyte migration, with activation of SHIP-1 (and subsequent inhibition of PI3K) and decreased expression of CXCR3 two mechanisms through which this may occur (Figure 5.22).

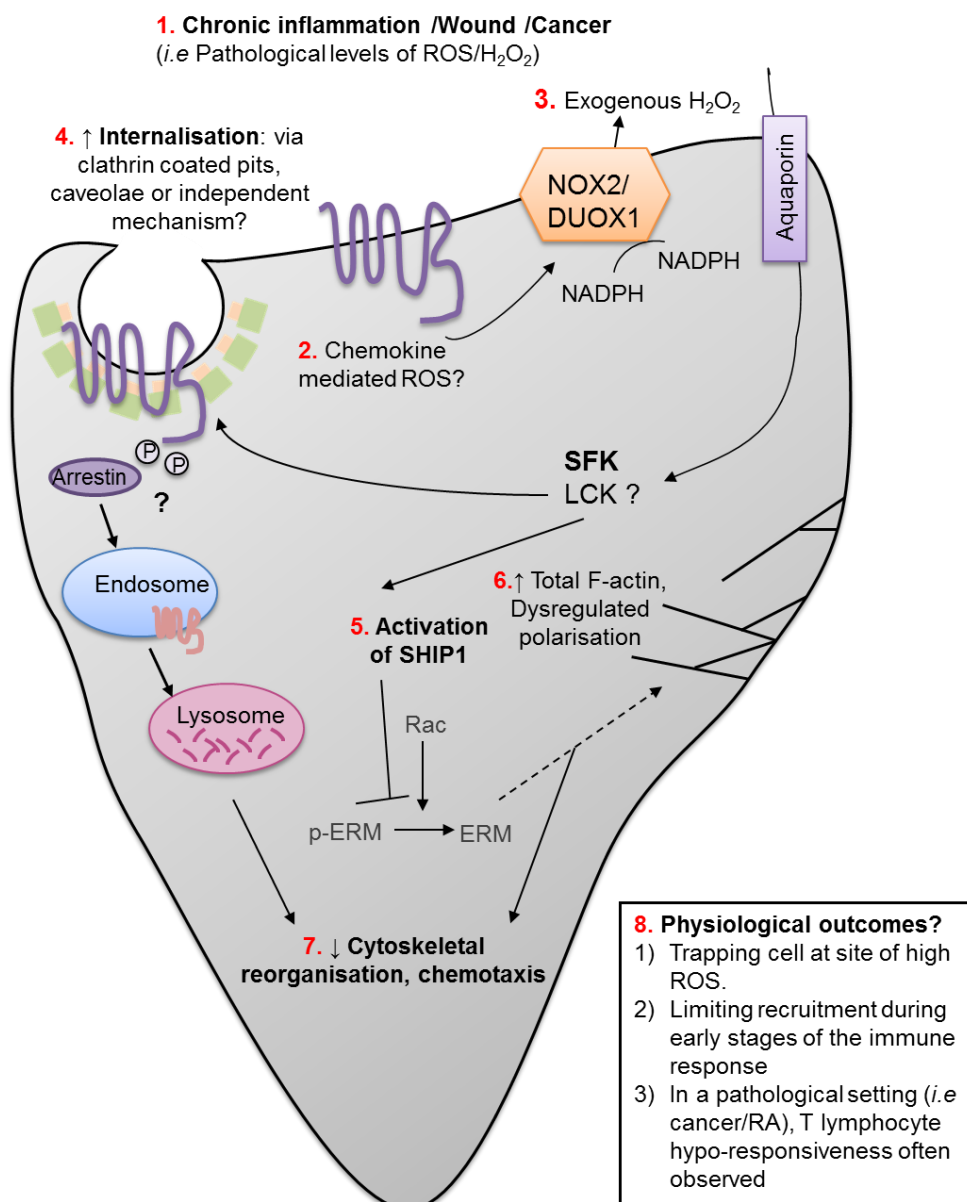


Figure 5.22 Model of mechanisms underlying ROS-induced migratory defect in T lymphocytes. This model is based on the results of this thesis and some of the findings of (Kwon *et al.*, 2010; Hara-Chikuma *et al.*, 2012). **1)** Several pathologies result in accumulation of high levels of ROS including wounds, chronic infection and cancer. **2)** Chemokine stimulation also produces exogenous H₂O₂ through NOX2/DUOX enzymes, which are known to be expressed on T lymphocytes. **3)** Signalling concentrations of exogenous H₂O₂ enters the cell through aquaporin channels and can manipulate proteins in the cytosol. **4)** H₂O₂ signals through SFKs to internalise chemokine receptor CXCR3, internalisation could occur through arrestin recruitment/ clathrin coated pits, caveolae or an independent mechanism. Once internalised, CXCR3 is degraded in the endosome. **5)** H₂O₂ also signals through SFKs to phosphorylate and activate SHIP-1. SHIP-1 activation inhibits the ability of CXCL11 to de-phosphorylate cytoskeletal proteins ezrin, radixin and ERM and **6)** inhibits actin polarisation. H₂O₂ also increases total F-actin. **7)** Collectively, chemokine internalisation, SHIP-1 activation and actin dysregulation reduce cytoskeletal reorganisation and cell chemotaxis. **8)** Proposed outcomes of migration deficiency in T lymphocytes.

CHAPTER 6. General discussion

6.1 SHIP-1 in T lymphocyte biology

Overview

This thesis began by examining how SHIP-1, a negative regulator of PI3K, operates in human T lymphocyte biology. The role of PI3Ks in cell function has been extensively investigated in recent years, particularly as they have shown much promise as drug targets in cancer (Fruman *et al.*, 2009; Foster *et al.*, 2012; So *et al.*, 2012b; Okkenhaug, 2013). However, as yet inhibitors have failed to have significant impact in the treatment of autoimmune and inflammatory diseases. SHIP-1 now stands at a crossroads, with the first activator of SHIP-1 entering Phase III clinical trials for the treatment of asthma. However, its functions remain less well understood in human T lymphocytes.

I used a SHIP-1 activator and SHIP-1 inhibitor in combination with *in vitro* assays to allow side by side evaluation of SHIP-1 roles in T lymphocyte biology. A considerable amount of data was generated with key findings indicating that SHIP-1 is crucial in the regulation of ligand-mediated signalling, proliferation, adhesion, cytoskeletal protein re-arrangement and migration of primary human T lymphocytes. The product of SHIP-1 ((PI3,4)P₂) is distinct from the phospholipid which is elevated by the direct antagonism of PI3K signalling (*i.e.* PI(4,5)P₂). In our hands, both activation and inhibition of SHIP-1 lead to the same functional outcomes in T lymphocytes, which suggests that both PI(3,4,5)P₃ and PI(3,4)P₂ are vital in T lymphocyte signalling (Figure 3.15). This is consistent with the “two PIP signalling hypothesis” described by Kerr which suggests PI(3,4,5)P₃ and PI(3,4)P₂ have independent signalling roles, with the requirement for each depending on the cell type and surrounding environment (Kerr, 2011). .

Whilst some of our findings were in line with data from murine models others, such as the decreased proliferation, ran contrary to the findings in mice, and serve to highlight the fact that there are often substantial differences between the immune systems of mice and humans. This indicates the importance of studying the role of proteins in human cells, even if the assays available are somewhat limited compared to animal models.

SHIP-1 now stands at a crossroads, with the first activator of SHIP-1 entering Phase III clinical trials. Importantly, although pre-clinical experiments have indicated that SHIP-1 modulation can alter TCR-induced response, no inappropriate T cell mediated autoimmunity or T cell neoplasm have thus far been demonstrated in either the pharmacological or genetic strategies implemented to manipulate SHIP-1. Work

identified in this thesis suggests SHIP-1 is a key role as a modulator of PI3K signalling in T lymphocytes and provides evidence to support using SHIP-1 modulators as treatments of inflammatory and autoimmune diseases.

In addition, there are potential benefits to targeting SHIP-1 over isoform specific PI3K inhibitors. Firstly, there is a large amount of functional redundancy and plasticity between PI3K isoforms, thus isoform targeted therapies might not warrant successful treatments in disease setting. Moreover, PI3K dysregulation is often caused by over-active mutations in PI3K (Pazarentzos *et al.*, 2015; Young *et al.*, 2015), these could alter the efficacy and affinity of PI3K inhibitors. Furthermore, the ubiquitous nature of PI3K signalling implies that any successful PI3K inhibitors could be complicated by non-selective effects and worryingly, it has recently been reported that resistance mechanisms can be developed to combat PI3K-targeted therapy (Ilic *et al.*, 2011); although selective pressure driving resistance could vary between cancer and inflammatory setting. SHIP-1 exhibits a restricted expression profile limiting non-selective effects and modulation of SHIP-1 appears to have proliferative effects on the immune system. SHIP-1 could be a superior target, and at the very least it will be beneficial to have multiple mechanisms to pharmacologically target PI3K signalling.

Future work

In light of investigations described in this thesis, future experiments should seek to address the following unanswered questions and hypotheses.

- Examine mechanisms underlying SHIP-1 modulators effect on proliferation. At what stage of the cell cycle is the proliferation inhibited?
- To identify the roles of SHIP-1 modulators upon the differentiation of T lymphocytes to different sub-sets.
- In a migrating cell, PI3K localises to the front of the cell and PTEN to the back to generate a polarised production of PI(3,4,5)P₃. Is SHIP-1 expression polarised during chemotaxis? As SHIP-1s product PI(3,4)P₂ has roles in lamellipodia formation and actin regulation through its downstream effector TAPP1 and Lpd, I hypothesise that SHIP-1s product PI(3,4)P₂ is not solely localised at the back of the cell. This could be determined using by expressing a fluorescent PI(3,4)P₂ reporter into T lymphocytes, such as fluorescently labelled PH domain of TAPP1. SHIP-1 localisation would then be observed under basal and ligand stimulated conditions.
- Our pharmacological approach could be applied to determine the functional effects of SHIP-1 activation or inhibition on T lymphocytes isolated from patients with T

lymphocyte component to their pathologies, such as asthma or COPD. Further supporting the use of these compounds as specific disease treatment.

6.2 ROS in human T lymphocyte biology

Overview

The second facet of this thesis sought to determine the functional and signalling roles of ROS in T lymphocyte biology. There is a rapidly growing consensus the oxidative signalling is important in diverse cell functions. At present, the roles of ROS in human T cell biology are under-investigated, particularly whether ROS affect the cells ability to migrate. Two models of inflammation have indicated the importance of H₂O₂ as a cue for innate immune cell migration to a wound (Niethammer *et al.*, 2009; Moreira *et al.*, 2010). Results which were duplicated in human neutrophils (Yoo *et al.*, 2012) and consistent with an observation that H₂O₂ uptake is required for mouse T lymphocyte migration to chemokine (Hara-Chikuma *et al.*, 2012). Therefore I hypothesised that H₂O₂ could act as a chemoattractant for human T lymphocytes. Specific results have already been discussed in the previous sections; a more detailed discussion of the possible implications and limitations of these studies will now follow.

ROS species are known to induce bifurcate responses within cells, with high concentrations evoking oxidative stress-induced cell death and low concentration required cell signalling and function. Therefore, the effects of pharmacological manipulation of ROS was examined with anti-oxidants used to lower the redox environment and a wide range of concentrations of H₂O₂ (1nM-10mM) to examine both the signalling roles and effect of oxidative stress on T lymphocyte biology. In addition to using a wide range of concentrations of H₂O₂ I could have also have examined a time course for H₂O₂ signalling, as although consistent, 30 minutes is a long exposure time to H₂O₂ in terms of its half-life. Dampening the redox environment with anti-oxidants decreased long term human T lymphocyte survival and proliferation, indicating that low levels of oxidants are required for T lymphocytes to function. Interestingly, instead of acting like a chemoattractant, low concentrations of H₂O₂ (1nM) appear to signal through SFK to selectively phosphorylate and increase the catalytic activity of SHIP-1 and down-regulate the expression of CXCR3, resulting in inhibition of T lymphocyte chemotaxis (Figure 5.22).

High concentrations of H₂O₂ activated cellular signalling including PI3K, MAPK, Src and PLC and intracellular Ca²⁺ elevation. However, although these are positive signalling

pathways that are known to be required for T cell survival and proliferation, it is likely that oxidative stress overwhelms the cellular antioxidants causing oxidative damage (Steinberg, 2013) resulting in our observations of dysregulated F-actin, decreased proliferation and survival of T lymphocytes (Figure 5.21).

Interestingly, quantification of protein thiol oxidation in cells has indicated that oxidation events are not equal across the cells, particularly at distinct cellular organelles (Balaban *et al.*, 2005; Go *et al.*, 2008). Instead of global signalling roles, oxidants appear to accumulate in cellular compartments to co-ordinate precise, local signalling events. It has been very difficult to determine the concentrations of ROS found in cells however, work has been done to determine the location of ROS production in different cell compartments using sub-cell-compartment targeted ROS sensitive GFP reporter expression. Distinct responses have been observed in different cell types (Go *et al.*, 2008; Waypa *et al.*, 2010) and this approach could be used to look at the accumulation of ROS in T lymphocytes.

I speculate that under physiological conditions ROS localised to small cellular compartments may accumulate to high concentrations, thus could signal through similar positive signalling pathways that were observed with global H₂O₂ exposure, without overwhelming cellular antioxidants and evoking cell death. An elegant approach would be to use these organelle specific H₂O₂ reporters to witness ROS accumulation in T lymphocytes downstream of ligand stimulation.

H₂O₂ is a very small, highly diffusible molecule with a short half-life due to its rapid reactivity and degradation. Therefore, as described above, the approach to add bolus application of H₂O₂ is unlikely to represent the intricacies of ROS signalling *in vivo*. The concentration of H₂O₂ was not monitored over the course of these experiments and therefore it is not known how rapidly it was degraded/ diffused. In particular, the bolus pre-treatment of H₂O₂ is unlikely to model the tissue-scale gradient of H₂O₂ that is generated at a wound site which extends between 100-200µm into the tail fin (Niethammer *et al.*, 2009). Therefore, a sustained gradient with continuous production of H₂O₂ might have a distinct effect upon T lymphocyte migration. It is however possible that bolus treatments of H₂O₂ might be more reflective of disease states, such as cancer where persistent oxidative stress is observed (Coussens *et al.*, 2002; Griffiths, 2005; Bartsch *et al.*, 2006; Murata *et al.*, 2014).

In addition, it has also been shown that bolus and continuous low level H₂O₂ exposure can lead to substantially different signal transduction and cellular responses (Sobotta *et al.*, 2013), with high bolus H₂O₂ reported to lead to artificial results. However, at present

it is still very difficult to reliably predict that physiological and pathological levels of ROS. Chemiluminescence methods have determined that H_2O_2 concentration in stimulated human neutrophils rarely exceed $10\mu\text{M}$ (Mueller *et al.*, 1995). In the zebrafish tailfin wounding model a maximal concentration of $50\mu\text{M}$ H_2O_2 was observed at the wound edge. Thus, these two differing concentrations indicate how difficult it would be to extrapolate the precise concentrations of ROS within the human body in each given scenario. Until these concentrations are known it is difficult to predict a bolus treatment concentration which can be described as “artificial”. However, both studies suggest that it is unlikely cells will be exposed to prolonged concentrations of H_2O_2 in the millimolar range.

Based on the information provided in this thesis it is clear that reactive oxygen species are not merely an unwanted by product of aerobic respiration which cause unwanted damage, instead are biological entities that are required for co-ordinating crucial physiological processes. The physiological environment of a T lymphocyte should be considered when planning *in vitro* studies investigating the biology of T lymphocytes. Importantly, the timing and subcellular localization of ROS generation are likely of greater influence in T cell responses than overall redox balance. Interestingly, controlled clinical trials have failed to show a consistent benefit of antioxidants in diseases setting (Rapola *et al.*, 1997; Yusuf *et al.*, 2000; Tang *et al.*, 2005). This supports our observation that oxidants are not solely toxic to the human body, that ROS are both protective and deleterious depending on concentration.

ROS potently inhibiting T lymphocyte migration was unexpected and has unearthed several interesting questions. What are the physiological consequences of low levels of ROS suppressing migration? There are several hypothetical scenarios (1) H_2O_2 could dampen the recruitment of adaptive immune cells which are required later in the wound repair process (2) in contrast, low level ROS could be responsible for capturing T lymphocytes at a site of high ROS by restricting their migration and (3) in a pathological setting, high ROS levels cause wide-spread suppression T lymphocyte migration and aid cancer cell survival or prolong chronic infection. In addition, a study by Hara-Chikuma *et al.* 2012 indicated that H_2O_2 uptake through aquaporin 3 is required for CXCL12-induced mouse T lymphocyte migration; why the disparity? This could be due to interspecies variation between mouse and human cells, and indicates that further investigation into the role of aquaporin 3 and H_2O_2 should be undertaken in human T lymphocytes.

Future directions

In light of investigations described in this thesis, future experiments should seek to address several un-answered questions and objectives:

- Determine whether ROS have a role in the differentiation of T lymphocyte subsets (i.e. Th1/Th2 or Th17 generation)
- Examine mechanisms underlying ROS manipulators effect on proliferation. At what stage of the cell cycle is the proliferation inhibited?
- Clarify whether protein phosphorylation is a good marker of oxidative activation.
- Design an *in vitro* migration assay which maintains a gradient of H₂O₂ and allows H₂O₂ to be detected over the time course of the experiment
- Determine the source of basal ROS that reduces T lymphocyte migration? (i.e. why is migration enhanced when cells are pre-treated with catalase), and are there any other functions of T lymphocytes which are enhanced by catalase treatment?
- Investigate the mechanism behind H₂O₂-induced internalisation of the CXCR3 receptor
- Use organelle specific, redox sensitive probes to determine the changes in ROS accumulation in real time within single organelles following ligand stimulation in T lymphocytes
- Examine the roles of hypoxic environment upon T lymphocyte functions (activation, differentiation, survival, migration to more chemokines). Furthermore, investigate the effect of manipulating ROS in a hypoxic environment
- Peroxynitrites also appear to have signalling roles within cells (Pacher *et al.*, 2007; Liaudet *et al.*, 2009). Characterise the roles of peroxynitrites in human T lymphocyte biology

6.3 Concluding remarks

T lymphocytes rely upon co-ordinated cellular signalling to undertake their physiological functions and resolve infections. However, these pathways often become dysregulated in states of pathology. Too much or too little signalling greatly perturbs the ability of T lymphocyte to function which can lead to hypo-responsiveness or hyper-activation. This thesis provides insight into how signalling pathways function in T lymphocytes. Understanding T lymphocyte signalling will lead to novel therapeutic strategies for T lymphocyte driven pathologies, including autoimmune/inflammatory disease and cancer.

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